

AD _____

MIPR NO: 93MM3513

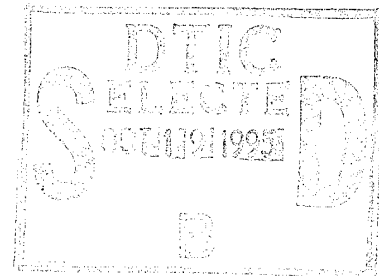
TITLE: Proposal for Research in Quantitative Bioassay Methodology and Risk Analysis and Characterization

PRINCIPAL INVESTIGATOR: Donald P. Gaver and Patricia A. Jacobs

CONTRACTING ORGANIZATION: Naval Postgraduate School
Monterey, California 93943

REPORT DATE: January 20, 1995

TYPE OF REPORT: Annual



PREPARED FOR: U.S. Army Medical Research and Materiel
Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 3

19951018 002

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 20 Jan 95	3. REPORT TYPE AND DATES COVERED Annual 11 Jan 94 - 11 Jan 95	
4. TITLE AND SUBTITLE Proposal for Research in Quantitative Bioassay Methodology and Risk Analysis and Characterization			5. FUNDING NUMBERS 93MM3513	
6. AUTHOR(S) Donald P. Gaver and Patricia A. Jacobs				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Postgraduate School Monterey, CA 93943			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) A summary of research conducted during the period January 12, 1994 – January 12, 1995 is given. Included are results of analyses of data obtained from other investigators supported by the U.S. Army Biomedical Research and Development Command. The data sets considered included data from a pilot study conducted by Gulf Coast Research Laboratory, various data sets from a cell proliferation study using medaka, female breast cancer data, and preliminary data from a health screen study for medaka. The analysis of experimental data has focused attention on the importance of controlling experimental conditions so as to minimize unwanted sources of variability. A preliminary mathematical model to describe the production of free radical O_2^- that is stimulated by the introduction of an initial dose of PMA at time zero is presented. A preliminary model for the occurrence and repair of cell adducts caused by dose stimulus is also presented.				
14. SUBJECT TERMS logistic regression; cross validation; goodness of fit; analysis of variance; mathematical models			15. NUMBER OF PAGES 188	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Accountable For	
ERIS GRAM	<input checked="" type="checkbox"/>
ERIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution	
Date	
Initials	
Date	

Ronald P. Gary 1/23/95
PI - Signature Date

QUICED AT GOVERNMENT EXPENSE

TABLE OF CONTENTS

I.	INTRODUCTION AND BACKGROUND	1
II.	APPROACHES TAKEN AND PROGRESS.....	1
	A. Analysis of Data from the Pilot Study in Medaka Conducted by Gulf Coast Research Laboratory	1
	B. Analysis of Data From a Cell Proliferation Study Using Medaka (Oryzias latipes).....	3
	C. Analysis of Female Breast Tissue Data in Order to Predict Cancer	4
	D. Analysis of Data Sent by Dr. L. Twerdok of GEO-CENTERS, INC. at U.S. Army BRDL in October 1994.....	6
	E. Mathematical Models	6
III.	CONCLUSIONS	7
	REFERENCES	8
	APPENDICES	
	1. An Exploratory Analysis of Data from a Mega-Medaka Study	1-1
	2. Assessment of Liver Modification and Cell Proliferation in Medaka Under DEN and TCE Concentration: Using Data Available 4/26/94	2-1
	3. Comparison of Area Indices in Medaka Livers for Sacrifices at Different Concentration and Time Combinations Using Data Available 5/19/94	3-1
	4. Modeling and Statistical Analysis of Bioassay Data: Medaka Cell Proliferation under DEN and TCE	4-1
	5. An Analysis of Female Breast Tissue Data In Order to Predict Cancer	5-1
	6. Remarks Resulting From a Preliminary Examination of Data Sent by Dr. L. Twerdok of GEO-CENTERS, INC. at U.S. Army BRDL in October 1994.....	6-1
	7. Mathematical Modeling of Free Radical $O_2^{\cdot -}$ Production Stimulation by PMA	7-1
	8. Models for Adduct Damage Fixation in the Presence of Repair	8-1

Proposal for Research in Quantitative Bioassay Methodology, and Risk Analysis and Characterization

I. INTRODUCTION AND BACKGROUND

The objectives of the above project were formulated in discussion with Mr. Henry Gardner of U.S. Army Medical R&D Command, Ft. Detrick, Maryland. The project purpose and workscope was stated in the proposal as follows: to perform mathematical, statistical and risk-analytical work in support of the mission of the Army Biomedical Research and Development Laboratory (ABRDL).

II. APPROACHES TAKEN AND PROGRESS

We have analyzed data obtained from other researchers supported by ABRDL. We have also developed preliminary mathematical models to summarize experimental findings by ABRDL-supported researchers. Brief descriptions of our work are given below.

A. Analysis of Data from the Pilot Study in Medaka Conducted by Gulf Coast Research Laboratory

Dr. Marilyn G. Wolfe of Experimental Pathology Laboratories, Inc. sent us summary incidence tables for selected liver neoplasms from a pilot study in medaka conducted by Gulf Coast Research Laboratory. The study was entitled "Dose response relationships for hepatocarcinogenesis in medaka (*Oryzias latipes*) exposed to waterborne N-Nitrosodiethylamine [DEN]."

The data analysis reported in Appendix 1 uses the summary table of incidences of hepatocellular neoplasms (adenoma[s] and/or carcinoma[s]) combined. There are 5 treatment groups in the study: a control, and those with concentration levels of

2.5 mg/L, 5.0 mg/L, 10.0 mg/L, and 20.0 mg/L. Each treatment group is assigned 4 tanks of medaka. There is one experiment which uses medaka that are 6 days of age at the start of the experiment and another experiment which uses medaka that are 52 days of age at the start of the experiment. A number of fish from each tank are sacrificed at 4, 6, and 9 months. The livers are examined and the number of fish exhibiting hepatocellular neoplasms (adenomas[s] and/or carcinoma[s]) is recorded.

Graphical displays of the data suggest that the log odds of at least one neoplasm occurring is roughly linearly increasing with concentration and time of sacrifice. The displays also suggest that the age of the medaka at the start of the experiment has an effect. The medaka that are of age 6 days at the start of the experiment tend to have higher incidence of at least one neoplasm than those of age 52 days at the beginning of the experiment. This suggests that young fish have greater concentration-response sensitivity than do old fish. It may recommend the use of young fish as toxin biological detectors.

Data with covariates and binary responses are often usefully described using a logistic regression model cf. Collett (1991). If $x_{i,1}, \dots, x_{i,n}$ denote the values of covariates (e.g. sacrifice times, concentration) for the i^{th} animal, then

$$P\{i^{\text{th}} \text{ animal has at least one neoplasm} | x_{i,1}, \dots, x_{i,n}\} \\ = [1 + \exp\{\beta_1 x_{i1} + \beta_2 x_{i2} + \dots + \beta_n x_{in}\}]^{-1}.$$

Appendix 1 describes the results of using the logistic regression model to explore incidences of hepatocellular neoplasms as a function of concentration and sacrifice time.

A statistical model can not only be used to describe data but also be used to predict data. Appendix 1 also presents results which explore the ability of certain logistic regression models estimated with one part of the data to predict the other part. Of particular interest are models estimated with higher concentration data and used to predict lower concentration results; and models estimated with data from small sacrifice

times and used to predict data from larger sacrifice times. The results suggest that the models have some ability to predict. However, additional data at small sacrifice times and smaller concentrations are needed to improve the accuracy and usefulness of the statistical models. Careful choice of concentration levels can be expected to improve the quality of the low-concentration response predictions.

B. Analysis of Data From a Cell Proliferation Study Using Medaka (*Oryzias latipes*)

Appendices 2–4 report data analyses of an experiment using Japanese medaka to study cell proliferation under different concentrations of DEN and TCE. Appendices 2–3 report analyses of summary data.

The medaka are exposed to differing levels of DEN and TCE. Each treatment group has two replicate tanks. Eight animals in each tank were sacrificed on 4 August 1993; this is sacrifice B. Eight additional animals in each tank were sacrificed on 20 August 1993; this is sacrifice D. Each sacrificed fish was exposed to BrdU for 72 hours prior to sacrifice; any cell that is in S-phase during this time has a BrdU marker. Each sacrificed fish is frozen and sliced longitudinally into 7-micron sections.

Five slices containing a portion of liver are considered for each fish. An agent was used that stains the nuclei with the BrdU marker black; these nuclei are called *positive*. A region of interest (ROI) is marked on the slice; the ROI is chosen so as to maximize the number of hepatocytes and minimize the number of nonhepatocytes present.

For half the fish in sacrifice B two measures of cell proliferation were estimated; a *count index* (CI) and an *area index* (AI). The count index for a slice is the number of positive hepatocytes in the ROI divided by the number of hepatocytes in the ROI, multiplied by 100. The evaluation of the latter measure is very labor-intensive. An alternative is an area index (AI) which is (the area of positive nuclei in the ROI divided by the area of the hepatocytes in the ROI) times 100. The AI is easier to obtain; however, it does not quantify cells in S-phase exactly as does the CI since cells can be of different size. Graphical analysis indicates that there appears to be a satisfactory linear

relationship between count index and area index, indicating that AI and CI are generally measuring the same response. However, the variability of the area index increases as the count index increases; this increase is generally associated with high DEN and TCE concentration levels. This association suggests that cell sizes become more variable under concentration of DEN and TCE.

Data from the experiment arrived in three phases. Initially only summary data was available. Appendices 2–3 describe results of data analysis using summary measures of area indices of 3–5 slices for each fish. Appendix 4 describes results of data analysis using the area indices of the slices for each fish. The results of the data analysis can be briefly, and simplistically, summarized as follows. While it can be said that there is a statistically significant difference between mean responses of (the square root of) the area index to the various treatments with DEN and TCE, no simple and interpretable dose-response patterns have been found. In particular, response does not appear to increase (or decrease) systematically with dose increase, where “dose” includes time of exposure as well as increases in chemical concentration levels. It remains to be seen whether the latter inconclusivity is lessened by the analysis of more data (later sacrifices), by finding that experimental problems or biases occurred, *or*, more exciting, that the dose-responses observed can be explained by biological mechanism, and that the findings essentially reappear when further experiments and data analyses are conducted.

C. Analysis of Female Breast Tissue Data in Order to Predict Cancer

In October 1994, Dr. D. Malins sent us data dated 10/25/94 from a study of female breast tissues. The data are measurements from breast tissue samples from 30 female patients. Fifteen of the patients underwent reduction mammoplasty; tissues from these patients are considered to be normal. The other 15 patients had invasive ductal carcinoma. The study used multiple breast tissue samples from some patients.

The data considered are measurements of fapyadenine (Fapy-A), 8-hydroxyadenine (8-OH-A), fapyguanine (Fapy-G), and 8-hydroxyguanine (8-OH-G) from breast tissue samples.

Appendix 5 reports the results of an analysis of data consisting of 68 samples from women who underwent reduction mammoplasty and 10 samples from invasive ductal tumors for a total of 78 samples. Of interest was the ability of the covariates Fapy-A, 8-OH-A, Fapy-G, and 8-OH-G to predict the occurrence of cancerous/normal tissue. Logistic regression models were used in the study.

One way to evaluate the usefulness of a statistical model is to evaluate how well it describes the data used to estimate the parameters of the model (goodness-of-fit). Another way is to evaluate how well the statistical model predicts new data that was not used to estimate its parameters. This latter process is called *cross validation*; it is a natural and well-accepted procedure for assessing the quality of a proposed prediction methodology. Mosteller and Tukey (1977) give a good discussion.

Simulation is used for cross validation. In each simulation replication, the data are randomly allocated to one of two data sets. One data set is used to estimate the parameters of a logistic regression model. The estimated model is then used to predict the probability that a data point from the other data set is from a cancerous tissue.

A summary of the results is as follows. Of the logistic regression models considered, the logistic regression model with the best goodness-of-fit is, not surprisingly, the one with the greatest number of covariates: constant, log (Fapy-A), log (8-OH-A), log (Fapy-G), and log (8-OH-G). However, this model tends to be the weakest predictor; this model can be viewed as overfitting the data. There are two logistic regression models that are better predictors: regression Model I has these covariates: constant, log (Fapy-A) and log (8-OH-A); the other logistic regression model (II) has these covariates: constant and log (Fapy-A/8-OH-A). Regression Model II tends to predict the occurrence of normal samples somewhat better than Model I does. However, Model I

predicts cancer samples somewhat better than Model II. What this means is that Model I tends to give more false positives than Model II; whereas Model II tends to give more false negatives than Model I. All the logistic regression models considered had more difficulty in predicting the cancer samples than they did predicting the normal samples: on the average about 1 out of 5 cancer samples was incorrectly predicted.

D. Analysis of Data Sent by Dr. L. Twerdok of GEO-CENTERS, INC. at U.S. Army BRDL in October 1994

The data consist of measurements made on medaka that were sacrificed at different times. The information recorded for each fish include: the data of sacrifice; the age; the length in millimeters; the weight in milligrams; percent hematocrit; percent leukocrit; and the hatch date. These data are from the beginning of a study monitoring the health status of medaka used in toxicology studies.

Appendix 6 reports results of the data analysis. The analysis suggests that there are associations between the leukocrit measurement and the sacrifice date. This association may be due to the procedures used to measure leukocrit; it may also be due to differences in water quality and other physical factors in the experiment. The presence of a "sacrifice effect" may detrimentally affect the ability to measure effects of treatments across sacrifices. Sacrifice effects will tend to dilute the strength of associations between measured variables and treatments.

E. Mathematical Models

Appendix 7 presents preliminary simple mathematical models to describe, in a quantitative way, the behavior of data exhibited by Dr. Judith Zelikoff at the U.S. ABRDL Research Project Review held on September 20–21, 1994. The models describe the production of free radical $O_2^{\cdot -}$ that is stimulated by the introduction of an initial concentration of PMA at time zero. The production is made visible or observable by surrounding the cells exposed to PMA with a solution of luminal. These models are a first step to providing a quantitative concentration-response relationship. The

mathematical models could also be used to simulate experiments before they are done. Such simulations could improve the design and cost-effectiveness of the experiments.

Appendix 8 presents a preliminary model for the occurrence and repair of cell adducts caused by a dose stimulus. This model was motivated by discussions with Dr. J. G. Burkhart of the Environmental Toxicology program, National Institute of Environmental Health Science. The model is also relevant to the analysis of female breast data from Dr. D. Malins.

We propose to spend more effort on such model-construction and exploration in future. Incorporation of biological mechanism into dose-response relationships is expected to provide better predictions of toxic dose effect than does the use of simple standard statistical methods.

III. CONCLUSIONS

During the past 12 months we have participated in the analysis of data obtained from other investigators supported by ABRDL and initiated the development of mathematical models in collaboration with some investigators to summarize and predict data in a more meaningful way.

The analysis of experimental data has focused attention on the importance of controlling experimental conditions so as to minimize unwanted sources of variability such as tank effects and time of sacrifice effects. Unless these sources of variability are controlled or adjusted for, they will tend to dilute the strength of inferred associations between measured variables and treatments. The study initiated by Dr. Twerdok should be helpful in controlling these unwanted sources of variability. The design of experiments should also be sensitive to these sources of variability.

The usefulness of a statistical model can be evaluated in two ways. One is to evaluate how well it describes the data used to estimate the parameters of the model (goodness-of-fit). Another way is to evaluate how well the statistical model predicts

new data that is not used to estimate its parameters (cross validation). It is important to incorporate this latter evaluation in the analysis of data.

Mathematical and statistical models are important not only because they summarize current data but also because they can be used to predict. For example, they can assist in planning future experiments. A model can assist in determining the number of animals needed and the number of sacrifice times and when they should occur to estimate a dose response relationship.

We propose to continue to perform mathematical, statistical and risk-analytical work in support of ABRDL.

REFERENCES

Collett, D. *Modeling Binary Data*. Chapman & Hall, London, 1991.

Mosteller, F. and Tukey, J. W. *Data Analysis and Regression*, Addison-Wesley, Reading, MA, 1977.

Twerdok, L. E. "Monitoring the health status of medaka used in toxicological studies." Presentation at Sixth Annual Research Review, U.S. Army Biomedical Research and Development Laboratory, 20-21 September 1994.

APPENDIX 1

An Exploratory Analysis of Data from a Mega-Medaka Study

by

D. P. Gaver

P. A. Jacobs

1. Introduction: Experimental Situation

This paper reports the results of an exploratory data analysis of the combined incidences of hepatocellular neoplasms and carcinomas from the summary incidence tables for selected liver neoplasms from the pilot study in medaka conducted by Gulf Coast Research Laboratory. The study was entitled "Dose response relationships for hepatocarcinogenesis in medaka (*Oryzias latipes*) exposed to waterborne N-Nitrosodiethylamine [DEN]." The data used for the data analysis reported here appear as Appendix C.

There are 5 treatment groups in the study: a control, and those with concentration levels of 2.5 mg/L, 5.0 mg/L, 10.0 mg/L and 20.0 mg/L. Each treatment group is assigned 4 tanks of medaka. There is one experiment which uses medaka that are 6 days of age at the start of the experiment and another experiment which uses medaka that are 52 days of age at the start of the experiment. A number of fish from each tank are sacrificed at 4, 6 and 9 months. The livers are examined for hepatocellular neoplasms (adenomas and/or carcinomas). The number of fish exhibiting neoplasms is recorded. Apparently several pathologists assessed fish condition; we analyzed data from one such assessment (or a consensus thereof).

Sections 2 and 3 describe a graphical analysis of the data. Section 4 describes the results of using a logistic regression model to estimate parameters. One

would hope that a statistical model would not only describe data well but could also be used to predict. In Sections 5–7 we explore the predictive ability of logistic regression models estimated using part of the data to predict the other part of the data.

2. A Graphical Exploratory Analysis

Figures 1 and 2 (respectively 3 and 4) display data from the experiment using medaka of age 6 days (respectively 52 days) at the start of the experiment.

Figure 1 (respectively Figure 3) displays 3 scatter plots for medaka of age 6 days (respectively 52 days) at the start of the experiment: one for each sacrifice time. The plots are of the fraction of sacrificed medaka with at least one neoplasm versus concentration. A comparison of the scales on the two figures suggests that there is an association between the age of the medaka at the start of the experiment and the response to concentration and time of sacrifice. The medaka of age 6 at the start of the experiment tend to have a greater incidence of neoplasms. The association of neoplasms with concentration for the age 6 medaka appears to change with sacrifice time; convex for sacrifice times 4 and 6 months and concave for sacrifice time 9 months. Larger fractions are associated with larger concentrations and sacrifice times.

Figure 2 (respectively Figure 4) displays 5 scatter plots for medaka of age 6 days (respectively 52 days) at the start of the experiment; one for each concentration of the fraction of medaka; the plots are of the fraction of medaka with at least one neoplasm versus sacrifice time. A comparison of the figures once again suggests an association between age of the medaka at the beginning of the experiment and concentration. The association between sacrifice time and fraction of medaka with the abnormalities appears weaker for lower concentrations.

3. Logistic Model

The following logistic model is used for the exploratory data analysis. Suppose M fish are sacrificed at time t and let N be the number of fish whose livers have neoplasms. We assume that N has a binomial distribution with M trials and probability of at least one neoplasm occurring, (possibly) depending on covariates $\mathbf{x} = (x_1, x_2, \dots, x_c)$ such as the concentration and time of sacrifice; that is,

$$P\{N = n\} = \binom{M}{n} p(\mathbf{x})^n (1 - p(\mathbf{x}))^{M-n}, \quad n = 0, 1, \dots, M \quad (3.1)$$

with

$$p(\mathbf{x}) = \frac{1}{1 + e^{\beta\mathbf{x}}} \quad (3.2)$$

where

$$\beta\mathbf{x} = \sum_j \beta_j x_j. \quad (3.3)$$

Note that for this model the log odds of a neoplasm not occurring is

$$\theta(\mathbf{x}) \equiv \log \frac{1 - p(\mathbf{x})}{p(\mathbf{x})} = \beta\mathbf{x}; \quad (3.4)$$

$\theta(\mathbf{x})$ is called the logistic transform. Thus, plots of the data version of the log odds of a neoplasm not occurring versus potential covariates will give some idea of the functional relationship between the covariates and the probability of no neoplasm occurring.

The (empirical) log odds of a neoplasm not occurring is

$$\theta_i = \log \frac{n_i - o_i + \frac{1}{2}}{o_i + \frac{1}{2}} \quad (3.5)$$

is computed for each tank, each sacrifice time, and each concentration where n_i is the number of medaka sacrificed and o_i is the number of sacrificed medaka having at least one neoplasm.

Figures 5–8 display plots of the θ_i 's versus time of sacrifice and concentration. Figures 5–6 display the plots for medaka aged 6 days at the beginning of the experiment. Figures 7–8 display plots for medaka aged 52 days at the beginning of the experiment.

Figure 5 (respectively Figure 7) displays 5 scatter plots for medaka of age 6 days (respectively 52 days) at the start of the experiment. The scatter plots are of the log odds of a neoplasm not occurring versus time of sacrifice, one scatter plot for each concentration; there is one point for each tank. The plots indicate that the log odds of a neoplasm not occurring is roughly linearly decreasing with time of sacrifice.

Figure 6 (respectively Figure 8) displays 3 scatter plots for medaka of age 6 days (respectively 52 days) at the start of the experiment. The scatter plots are of the log odds of a neoplasm not occurring versus the concentration, one scatter plot for each sacrifice time; there is one point for each tank. The plots indicate that the log odds of a neoplasm not occurring is roughly linearly decreasing with concentration.

4. Logistic Analysis Conditional on Age, and By Concentration

Table 1 presents results of an analysis of the experiment in which the fish were of age 6 days at the start of the experiment. The data for each tank are used to fit a logistic model with the probability a fish develops at least one neoplasm by t time units into the experiment of the form

$$p(t) = \frac{1}{1 + e^{\beta_0 + \beta_1 t}}. \quad (4.1)$$

Table 1 displays the estimates of β_0 and β_1 for each tank in each treatment group and the estimates of β_0 and β_1 obtained by combining all the tanks in each treatment group. Also displayed are the standard errors of the estimates as computed using asymptotic Fisher Information. This is a standard biostatistical methodology. Within each treatment group, the four tanks are labeled 1 through 4. An alternative, re-sampling, or "the bootstrap method", might well provide more reliable results. Note that as the concentration increases the estimates of β_0 tend to decrease. Thus, the estimated probability of at least one neoplasm occurring at or before a fixed time t will tend to increase as the concentration increases, as is not surprising. Note also that the estimates of β_1 are negative. Thus, the estimated probability of at least one neoplasm occurring before time t will tend to increase as t increases. The estimated values of β_1 do not appear to depend on the size of the concentration (other than the control).

5. Logistic Model: Time and Concentration as Covariates; Low-Concentration Prediction

A logistic model with covariates sacrifice time and concentration is estimated using all data at concentrations 5.0, 10.0 and 20.0 mg/L; specifically, the model is that the probability a fish develops at least one neoplasm by t time units into the experiment with concentration d has the form

$$p(t;d) = \frac{1}{1 + e^{\beta_0 + \beta_1 t + \beta_2 d}}. \quad (5.1)$$

For medaka that are 6 days of age at the start of the experiment the estimates (asymptotic standard errors) [bootstrap standard errors] are

$\hat{\beta}_0 = 6.43$	$\hat{\beta}_1 = -0.65$	$\hat{\beta}_2 = -0.17$
(0.46)	(0.054)	(0.02)
[0.49]	[0.057]	[0.02]

This estimated model can be used to predict the probability of a neoplasm occurring before a sacrifice time at the smaller concentration 2.5 mg/L and the control. These predicted probabilities appear in Table 2 along with an estimated standard error. Also appearing is the fraction of medaka in all the tanks at concentration 2.5 mg/L which have at least one neoplasm; also displayed are the fractions of medaka in the control having neoplasms.

For medaka that are 52 days of age at the start of the experiment, the estimates (standard errors) using all data at concentrations 5.0, 10.0, 20.0 mg/L are

$$\begin{array}{ccc} \hat{\beta}_0 = 6.03 & \hat{\beta}_1 = -0.45 & \hat{\beta}_2 = -0.12 \\ (0.48) & (0.05) & (0.02) \\ [0.48] & [0.05] & [0.02] \end{array}$$

This estimated model can be used to predict probabilities of at least one neoplasm occurring by time t at the smaller concentration 2.5 mg/L and the control. These predicted probabilities appear in Table 3 along with an estimated standard error. Also displayed is the actual fraction of medaka that were found to have at least one neoplasm.

Both estimated models resulted in predicted probabilities for the control that are larger than the observed fraction of medaka having at least one neoplasm in each control group, particularly at the long exposure time. However, the standard errors of the predictions are also large. The predicted probabilities for the 2.5 mg/L groups are closer to the observed fraction for the experiment using medaka of age 52 days at the start of the experiment.

Table 4 (respectively Table 5) reports results of predicting the fraction of medaka in the control group that have neoplasms using a model estimated with data from the experiment with concentrations 2.5, 5.0, 10.0 and 20.0 mg/L; the

experiment is for medaka aged 6 days (respectively aged 52 days) at the start of the experiment. The predicted fraction for the medaka sacrificed at 9 months is high.

6. Logistic Model: Time and (Concentration \times Time) as Covariates; Low-Concentration Prediction

A logistic model with covariates sacrifice time and concentration \times (sacrifice time) is estimated using all data at concentrations 5.0, 10.0, and 20.0 mg/L; specifically, the model is that the probability a fish develops at least one neoplasm by time t units into the experiment with concentration d has the form

$$p(t; d) = \frac{1}{1 + e^{\beta_0 + \beta_1 t + \beta_2 (t \times d)}}. \quad (6.1)$$

For medaka that are 6 days of age at the start of the experiment the estimates (asymptotic standard errors)

$$\begin{array}{ccc} \hat{\beta}_0 = 4.48 & \hat{\beta}_1 = -0.35 & \hat{\beta}_2 = -0.027 \\ \text{(s.e.)}(0.35) & (0.05) & (0.003) \end{array}$$

This estimated model can be used to predict the probability of a neoplasm occurring before a sacrifice time at the smaller concentration 2.5 mg/L and the control. These predicted probabilities appear in Table 2a along with the bootstrap standard error. Also appearing is the fraction of medaka in all the tanks at concentration 2.5 mg/L which have at least one neoplasm; also displayed are the fractions of medaka in the control having neoplasms.

For medaka that are 52 days of age at the start of the experiment, the estimates (standard errors) using all data at concentrations 5.0, 10.0, 20.0 mg/L are

$$\begin{array}{ccc} \hat{\beta}_0 = 4.35 & \hat{\beta}_1 = -0.22 & \hat{\beta}_2 = -0.017 \\ \text{(s.e.)}(0.37) & (0.06) & (0.002) \end{array}$$

This estimated model can be used to predict probabilities of at least one neoplasm occurring by time t at the smaller concentration 2.5 mg/L and the control. These predicted probabilities appear in Table 3a along with an estimated standard error. Also displayed is the actual fraction of medaka that were found to have at least one neoplasm.

The predictions with the model (6.1) are about the same as those for the logistic model with covariates concentration and time of sacrifice.

7. Logistic Model: Time as Covariate; High-Sacrifice Time Prediction

A logistic model with probability of at least one neoplasm occurring before time t of the form

$$p(t) = \frac{1}{1 + e^{\beta_0 + \beta_1 t}} \quad (7.1)$$

is estimated using data for fish sacrificed at $t = 4, 6$ months for each of the concentrations 2.5, 5.0, 10.0, and 20.0 mg/L. The resulting models are then used to predict the probability for $t = 9$ months. The results using data from the experiment with fish of age 52 days at the start of the experiment appear in Table 6. The predicted probabilities are always larger than the observed fraction.

8. Logistic Models with Other Functional Forms for the Covariates

Table 7 displays results of using the model

$$P(\text{develop neoplasm}) = \frac{1}{1 + e^{a + b \log(d \times t)}} \quad (8.1)$$

estimated using data for 5 mg/L, 10 mg/L and 20 mg/L to predict the fraction of medaka with at least one neoplasm for 2.5 mg/ ℓ treatment group; the experiment using fish of age 6 days at the start of the experiment is used. The model under-predicts the fraction.

Table 8 displays results of using the model

$$P(\text{develop neoplasm}) = \frac{1}{1 + e^{\beta_1 + \beta_2 \log t + \beta_3 \log d}} \quad (8.2)$$

estimated using data for 5.0 mg/L, 10 mg/L and 20 mg/L to predict the fraction of medaka with at least one neoplasm for the 2.5 mg/ ℓ treatment group, using the experiment started with fish 6 days of age. The predicted fractions are within 2 standard errors of the observed.

9. Summary

Incidences of hepatocellular neoplasms (adenomas and/or carcinomas) as a function of concentration and sacrifice time in the mega-medaka study are explored. Graphical displays suggest that the log odds of at least one neoplasm not occurring is roughly linearly decreasing with concentration and time of sacrifice. Graphical displays also suggest that the age of the medaka at the start of the experiment has an effect. The medaka that are of age 6 days at the start of the experiment tend to have higher incidence of at least one neoplasm than those of age 52 days at the beginning of the experiment.

The logistic regression model is used to explore incidences of hepatocellular neoplasms as a function of concentration and sacrifice time. The estimated logistic regression models for fish of age 6 indicate that a positive concentration increases the probability of at least one neoplasm occurring. A positive concentration also increases the probability of at least one neoplasm developing as the time of sacrifice increases. However, there is no apparent association between the size of the concentration and the probability increases as a function of sacrifice time.

A statistical model can not only be used to describe data but also be used to predict data. We explore the ability of logistic regression models with different

covariates estimated with one part of the data, to predict the other part. A summary of the results follows.

The prediction of the fractions of medaka with at least one neoplasm at 2.5 mg/L concentration and the control using logistic regression models estimated using the data from higher concentrations is explored; the model covariates are concentration and time of sacrifice. The prediction results using logistic models are better for 2.5 mg/L than those for the control. The prediction results for smaller sacrifice times are better than those for larger sacrifice times. The predicted fractions tend to be larger than the estimated fractions for the covariates of concentration and time of sacrifice. Models with covariates (time of sacrifice) and (concentration \times time of sacrifice) were also considered. The prediction results were similar.

The prediction of the fraction of medaka developing at least one neoplasm before the sacrifice time at $t = 9$ using a model fit using other data is explored; the covariates are concentration and time of sacrifice. The models fit using data from positive concentrations do not predict the fraction from the control group well; the models tend to overpredict the fraction. The models fit using data for $t = 4, 6$ within a concentration also tend to overpredict the fraction at $t = 9$.

The prediction of the fraction of fish developing at least one neoplasm at 2.5 mg/L concentration using a logistic model with covariates log concentration and log time of sacrifice estimated with data at larger concentrations is explored using the experiment started with fish 6 days old. The predicted fractions are smaller than the observed fractions.

REFERENCE

- Cox, D. R., *Analysis of Binary Data*, Chapman and Hall, 1970.
IBM Corporation. A Graphical Statistical System (AGSS).

Table 1

Age 6

Model: The number of fish with neoplasms has a binomial distribution with probability

$$p(t) = \frac{1}{1 + e^{\beta_0 + \beta_1 t}}$$

where t is the time of sacrifice

Estimates $\hat{\beta}_0$ $\hat{\beta}_1$

(std errors (Fisher Information))

Tanks Treatment	1			2			3			4			All	
	β_0	β_1		β_0	β_1		β_0	β_1		β_0	β_1		β_0	β_1
control	11.5 (93)	-0.11 (14)		25.7 (73)	-2.7 (8.1)		11.6 (94)	-0.14 (14)		11.0 (90)	-0.02 (13)		28.6 (107)	-2.8 (11)
fraction of medaka	$\frac{0}{25}$	$\frac{0}{25}$	$\frac{0}{14}$	$\frac{0}{24}$	$\frac{0}{24}$	$\frac{2}{11}$	$\frac{0}{25}$	$\frac{0}{25}$	$\frac{0}{12}$	$\frac{0}{25}$	$\frac{0}{25}$	$\frac{0}{23}$		
2.5 mg	6.1 (2.1)	-0.53 (0.27)		3.4 (1.4)	-0.22 (0.21)		6.8 (1.9)	-0.71 (0.22)		29.7 (114.8)	-3.2 (12.8)		6.0 (0.92)	-0.56 (0.12)
	$\frac{0}{25}$	$\frac{2}{25}$	$\frac{3}{16}$	$\frac{0}{24}$	$\frac{6}{25}$	$\frac{1}{11}$	$\frac{1}{25}$	$\frac{1}{25}$	$\frac{8}{19}$	$\frac{0}{25}$	$\frac{0}{25}$	$\frac{6}{21}$		
5.0 mg	8.8 (2.9)	-0.90 (0.35)		5.5 (1.3)	-0.64 (0.18)		4.4 (1.2)	-0.47 (0.17)		6.4 (1.4)	-0.81 (0.20)		5.6 (0.71)	-0.63 (0.09)
	$\frac{0}{25}$	$\frac{1}{25}$	$\frac{6}{19}$	$\frac{2}{24}$	$\frac{3}{25}$	$\frac{11}{18}$	$\frac{1}{25}$	$\frac{6}{25}$	$\frac{6}{14}$	$\frac{0}{25}$	$\frac{6}{24}$	$\frac{10}{15}$		
10.0 mg	5.0 (1.18)	-0.66 (0.172)		4.1 (1.05)	-0.62 (0.17)		6.0 (1.4)	-0.76 (0.20)		4.2 (1.10)	-0.66 (0.18)		4.58 (0.56)	-0.64 (0.09)
	$\frac{3}{25}$	$\frac{5}{25}$	$\frac{12}{16}$	$\frac{4}{25}$	$\frac{11}{25}$	$\frac{13}{16}$	$\frac{0}{25}$	$\frac{7}{25}$	$\frac{9}{14}$	$\frac{3}{25}$	$\frac{13}{25}$	$\frac{11}{14}$		
20.0 mg	6.3 (1.6)	-1.3 (0.33)		2.2 (0.95)	-0.42 (0.16)		5.7 (1.6)	-1.2 (0.32)		2.0 (0.97)	-0.46 (0.17)		3.4 (0.58)	-0.69 (0.11)
	$\frac{7}{25}$	$\frac{21}{25}$	$\frac{18}{18}$	$\frac{8}{25}$	$\frac{16}{25}$	$\frac{10}{13}$	$\frac{6}{24}$	$\frac{19}{25}$	$\frac{7}{7}$	$\frac{11}{25}$	$\frac{18}{25}$	$\frac{14}{16}$		

The labeling of tank number in the table sets the smallest tank number in a treatment group equal to 1, ..., and the largest tank number in a treatment group equal to 4.

The fraction of medaka has as numerator the number of fish with combined hepatocellular neoplasms and as denominator the number of fish livers examined. The fractions from left to right for each tank are for 4 months sacrifice, 6 months sacrifice, and 9 months sacrifice.

Table 2

Predicted fractions of medaka with at least one neoplasm occurring by time t for control group and concentration 2.5 mg/L group using model estimated with data for higher concentrations.

Model with Covariates: Time of Sacrifice, Concentration

Medaka Aged 6 days at Start of Experiment

Time	Predicted Fraction for 2.5 mg/L treatment group from Estimated Model (standard error) [bootstrap s.e.]	Fraction of Medaka in treatment group 2.5 mg/L having the symptom (standard error)	Pred. Frac. for control from Est. Model (standard error) [bootstrap s.e.]	Fraction of Medaka in control having symptom (standard error)
4	0.03 (0.04) [0.02]	0.01 (0.01)	0.02 (0.01) [0.02]	0 –
6	0.11 (0.06) [0.04]	0.09 (0.04)	0.07 (0.03) [0.03]	0 –
9	0.46 (0.13) [0.08]	0.27 (0.05)	0.36 (0.08) [0.08]	0.03 (0.02)

Table 2a

Predicted fractions of medaka with at least one neoplasm occurring by time t for control group and concentration 2.5 mg/L group using a logistic regression model estimated using data for higher concentrations.

Model with Covariates: (Time of Sacrifice)

((Time of Sacrifice) \times Concentration)

Medaka Aged 6 days at Start of Experiment

Time	Predicted Fraction for 2.5 mg/L treatment group [bootstrap s.e.]	Fraction of Medaka in treatment group 2.5 mg/L having the symptom	Predicted Fraction for Control Group	Fraction of Medaka in Control Group having the symptom
4	0.06 [0.03]	0.01 (0.01)	0.04 [0.02]	0 –
6	0.12 [0.03]	0.09 (0.04)	0.08 [0.03]	0 –
9	0.33 [0.07]	0.27 (0.05)	0.21 [0.07]	0.03 (0.02)

Table 3

Predicted fractions of medaka with at least one neoplasm occurring by time t for control and concentration 2.5 mg/L using model estimated with data for higher concentrations.

Model with Covariates: Time of Sacrifice and Concentration

Medaka Aged 52 days at Start of Experiment

Time	Predicted Fraction for 2.5 mg/L treatment group from Estimated Model (standard error) [bootstrap s.e.]	Fraction of Medaka in treatment group 2.5 mg/L having the symptom (standard error)	Pred. Frac. for control from Est. Model (standard error) [bootstrap s.e.]	Fraction of Medaka in control having symptom (standard error)
4	0.02 (0.01) [0.02]	0.01 (0.01)	0.014 (0.013) [0.01]	0.02 (0.01)
6	0.05 (0.02) [0.02]	0.04 (0.02)	0.03 (0.02) [0.02]	0 —
9	0.16 (0.05) [0.05]	0.14 (0.04)	0.12 (0.04) [0.04]	0.02 (0.01)

Table 3a

Predicted fractions of medaka with at least one neoplasm occurring by time t for control group and concentration 2.5 mg/L group using model estimated with data for higher concentrations.

Model with Covariates: (Time of Sacrifice)

((Time of Sacrifice) \times Concentration)

Medaka Aged 52 days at Start of Experiment

Time	Predicted Fraction for 2.5 mg/L treatment group from Estimated Model [bootstrap s.e.]	Fraction of Medaka in treatment group 2.5 mg/L having the symptom (standard error)	Pred. Frac. for control from Est. Model [bootstrap s.e.]	Fraction of Medaka in control having symptom (standard error)
4	0.04 [0.02]	0.01 (0.01)	0.03 [0.02]	0.02 (0.01)
6	0.06 [0.03]	0.04 (0.02)	0.05 [0.02]	0 –
9	0.12 [0.04]	0.14 (0.04)	0.08 [0.03]	0.02 (0.01)

Table 4

Predicted fraction of medaka with at least one neoplasm occurring by time t for the control using model estimated with data for positive concentrations.

Medaka Aged 6 days at Start of Experiment

time	Predicted Fraction for Control from Estimated Model (standard error) [bootstrap s.e.]	Fraction of Medaka in Control having the symptom (standard error)
4	0.02 (0.01) [0.01]	0 —
6	0.06 (0.03) [0.02]	0 —
9	0.28 (0.07) [0.07]	0.03 (0.02)

Model

$$P\{\text{at least one neoplasm by time } t\} = \frac{1}{1 + e^{\beta_0 + \beta_1 t + \beta_2 d}}$$

	β_0	β_1	β_2
Estimates	6.6	-0.63	-0.18
(std error)	(0.42)	(0.05)	(0.01)
[bootstrap s.e.]	[0.41]	[0.05]	[0.01]

Table 5

Predicted fraction of medaka with at least one neoplasm occurring by time t for the control using model estimated with data for positive concentrations.

Medaka Aged 52 days at Start of Experiment

time	Prediction Fraction of Control with neoplasms (standard error) [bootstrap s.e.]	Observed Fraction (standard error)
4	0.01 (0.01) [0.01]	0.02 (0.01)
6	0.03 (0.02) [0.02]	0 —
9	0.11 (0.04) [0.04]	0.02 (0.01)

	β_0	β_1	β_2
Estimates	6.16	-0.45	-0.12
(std error)	(0.43)	(0.05)	(0.01)
[bootstrap s.e.]	[0.44]	[0.05]	[0.01]

Table 6

Fish of Age 52 days at Start of Experiment

The model is

$$P(\text{develop neoplasm}) = \frac{1}{1 + e^{\beta_0 + \beta_1 t}}$$

Estimate parameters using data for fish sacrificed at $t = 4, 6$.

Predicted proportion of medaka with at least one neoplasm for those sacrificed at $t = 9$.

Concentration	Estimates		Predicted Fraction at $t=9$ (standard errors)	Fraction of Medaka sacrificed at $t=9$ with symptom (standard errors)
	β_0 (standard errors)	β_1 (standard errors)		
2.5	7.40 (3.2)	-0.70 (0.56)	0.25 (0.38)	0.14 (0.04)
5.0	31.9 (119) (all 0's at $t=4$)	-4.98 (19.8)	1.0 (0.0002)	0.13 (0.04)
10.0	7.16 (1.8)	-0.93 (0.32)	0.77 (0.20)	0.30 (0.05)
20.0	5.69 (1.19)	-0.82 (0.21)	0.84 (0.11)	0.61 (0.05)

Table 7

Predicted fraction of medaka with at least one neoplasm occurring by time t for 2.5 mg/L using model estimated with data for 5 mg/L, 10 mg/L and 20 mg/L.

Model

$$P(\text{abnormal}) = \frac{1}{1 + e^{a+b \log(d \times t)}}$$

	\hat{a}		\hat{b}	
	Logistic	Bootstrap	Logistic	Bootstrap
Estimates	9.35	9.37	-2.16	-2.16
(std error)	(0.71)	[0.70]	(0.17)	[0.16]

Medaka Aged 6 days at Start of Experiment

time	Predicted Fraction for 2.5 mg/L from Estimated Model [bootstrap s.e.]	Fraction of Medaka for 2.5 mg/L having the symptom
4	0.01 [0.01]	0.01
6	0.03 [0.01]	0.09
9	0.07 [0.03]	0.27

Table 8

Predicted fraction of medaka with at least one neoplasm occurring by time t for 2.5 mg/L using model estimated with data for 5 mg/L, 10 mg/L and 20 mg/L.

Model

$$P(\text{abnormal}) = \frac{1}{1 + e^{\beta_1 + \beta_2 \log t + \beta_3 \log d}}$$

Medaka Aged 6 days at Start of Experiment

	$\hat{\beta}_1$		$\hat{\beta}_2$		$\hat{\beta}_3$	
	Logistic	Bootstrap	Logistic	Bootstrap	Logistic	Bootstrap
Estimate	12.1	12.2	-4.07	-4.11	-1.90	-1.91
(std error)	(0.89)	[0.92]	(0.34)	[0.36]	(0.18)	[0.19]

Medaka Aged 52 days at Start of Experiment

	$\hat{\beta}_1$		$\hat{\beta}_2$		$\hat{\beta}_3$	
	Logistic	Bootstrap	Logistic	Bootstrap	Logistic	Bootstrap
Estimate	10.4	10.6	-3.01	-3.06	-1.4	-1.39
(std error)	(0.89)	[0.95]	(0.34)	[0.37]	(0.19)	[0.20]

Medaka Aged 6 days at Start of Experiment

Medaka Aged 52 days at Start of Experiment

Time	Predicted Fraction for 2.5 mg/L from Estimated Model [bootstrap s.e.]	Fraction of Medaka for 2.5 mg/L having the symptom (standard error)	Predicted Fraction for 2.5 mg/L from Estimated Model [bootstrap s.e.]	Fraction of Medaka for 2.5 mg/L having the symptom
4	0.009 [0.010]	0.01 (0.01)	0.007 [0.010]	0.01 (0.01)
6	0.05 [0.02]	0.09 (0.04)	0.02 [0.02]	0.04 (0.02)
9	0.20 [0.07]	0.27 (0.05)	0.07 [0.04]	0.14 (0.04)

Appendix A

Standard Errors for the Predicted Fraction of Medaka Which Have at Least One Neoplasm

1. Asymptotic Variance for Estimated Probability of at Least One Neoplasm Occurring

The logistic model for the probability of at least one neoplasm occurring is

$$\theta(\beta; x) = \frac{1}{1 + \lambda(\beta; x)}$$

where

$$\lambda(\beta; x) = \exp \left\{ \sum_{i=1}^p x_i \beta_i \right\}$$

with covariates (x_1, \dots, x_p) .

Assuming the model is correct, let β_i^0 be the true value of β_i .

An approximate asymptotic variance for $\lambda(\hat{\beta}; x)$ is based on the first two terms of a Taylor expansion

$$\begin{aligned} \lambda(\hat{\beta}; x) &\approx \lambda(\beta^0; x) + \sum_{j=1}^p \lambda(\beta^0; x) (x_j) (\beta_j - \beta_j^0) \\ \text{Var}[\lambda(\hat{\beta}; x)] &\approx E \left[\left(\sum_{j=1}^p \lambda(\beta^0; x) (x_j) (\beta_j - \beta_j^0) \right)^2 \right] \\ &= \sum_{j=1}^p \lambda(\beta^0; x)^2 x_j^2 \text{Var}(\hat{\beta}_j) + \sum_{j=1}^p \sum_{k=1, k \neq j}^p \lambda(\beta^0; x)^2 x_k x_j \text{Cov}(\hat{\beta}_j, \hat{\beta}_k) \\ &\approx \sum_{j=1}^p \lambda(\hat{\beta}; x)^2 x_j^2 \text{Var}(\hat{\beta}_j) + \sum_{j=1}^p \sum_{k=1, k \neq j}^p \lambda(\hat{\beta}; x)^2 x_k x_j \text{Cov}(\hat{\beta}_j, \hat{\beta}_k) \end{aligned}$$

An asymptotic variance for $\theta(\hat{\beta}; x)$ is

$$\begin{aligned}
 Var[\theta(\hat{\beta}; x)] &\approx \left(\frac{1}{(1 + \lambda(\hat{\beta}; x))^2} \right)^2 Var[\lambda(\hat{\beta}; x)] \\
 &\approx \left(\frac{\lambda(\hat{\beta}; x)}{(1 + \lambda(\hat{\beta}; x))^2} \right)^2 \left[\sum_{j=1}^p Var[\hat{\beta}_j] x_j^2 + \sum_{j=1}^p \sum_{k \neq j}^p x_j x_k Cov(\hat{\beta}_k, \hat{\beta}_j) \right] \\
 &= \left(p(\hat{\beta}; x) [1 - p(\hat{\beta}; x)] \right)^2 \left[\sum_{j=1}^p Var[\hat{\beta}_j] x_j^2 + \sum_{j=1}^p \sum_{k \neq j}^p x_j x_k Cov(\hat{\beta}_k, \hat{\beta}_j) \right]
 \end{aligned}$$

Asymptotic estimates of the variance and covariance of $\hat{\beta}_j$ can be obtained using Fisher Information.

2. Asymptotic Variance for Predicted Fraction of Sacrificed Fish with at Least One Neoplasm Occurring

To obtain a variance for the predicted fraction of sacrificed fish with neoplasms let M be the number of medaka sacrificed, N be the number of medaka having at least one neoplasm, and θ be the (unknown) probability of at least one neoplasm occurring by the time of sacrifice. The variance of the fraction of medaka that have one or more neoplasms is

$$\begin{aligned}
\text{Var}\left[\frac{N}{M}\right] &= E\left[\text{Var}\left[\frac{N}{M}\middle|\theta\right]\right] + \text{Var}\left[E\left[\frac{N}{M}\middle|\theta\right]\right] \\
&= E\left[\frac{1}{M^2} M\theta(1-\theta)\right] + \text{Var}[\theta] \\
&= \frac{1}{M}\left[E[\theta] - E[\theta^2]\right] + \text{Var}[\theta] \\
&= \frac{1}{M}\left[E[\theta] - \left\{\text{Var}[\theta] + E[\theta^2]\right\}\right] + \text{Var}[\theta] \\
&= \frac{1}{M}\left[E[\theta](1 - E[\theta])\right] + \text{Var}[\theta]\left(\frac{M-1}{M}\right).
\end{aligned}$$

Thus an (asymptotic) estimate of the variance of the predicted fraction of medaka having the symptom is

$$\begin{aligned}
\hat{\text{Var}}\left[\frac{N}{M}\right] &\approx \frac{1}{M}\theta(\hat{\beta};x)\left[1 - \theta(\hat{\beta};x)\right] \\
&\quad + \text{Var}\left[\theta(\hat{\beta};x)\right]\left(\frac{M-1}{M}\right).
\end{aligned}$$

Appendix B

The Parametric Bootstrap Experiment

Bootstrap assessments of estimate standard errors and predictive proportions use simulation. The original data $(m_i, n_i, x_{i1}, \dots, x_{ip})$ $i = 1, \dots, I$ are used to estimate the parameters of the logistic model; $\hat{\beta}_i^0$, $i = 1, \dots, p$. A bootstrap replication consists of the following. For each $i = 1, \dots, I$, a binomial random number b_i with m_i trials and probability of success $\left[1 + \exp\left\{\sum_{j=1}^p x_{ij} \hat{\beta}_j^0\right\}\right]^{-1}$ is drawn. The simulated data $(m_i, b_i, x_{i1}, \dots, x_{ip})$ are used to estimate the parameters of the logistic model, $\hat{\beta}_j^b$, $j = 1, \dots, p$. These bootstrap estimates are then used to predict the fraction of medaka that have neoplasms out of K fish sacrificed that have covariates y_1, \dots, y_p in the following manner. A binomial random number is drawn having K trials and probability of success $\left[1 + \exp\left\{\sum_{j=1}^p y_j \hat{\beta}_j^b\right\}\right]^{-1}$. The random number divided by the number of trials gives a bootstrap replication of the predicted fraction. This ends one bootstrap replication.

The bootstrap estimate of standard error of an estimate is the square root of the sample variance of the bootstrap estimate. The bootstrap standard error for the prediction proportion is the square root of the sample variance of bootstrap predicted fractions.

The bootstrap results reported use 500 bootstrap replications.

Appendix C

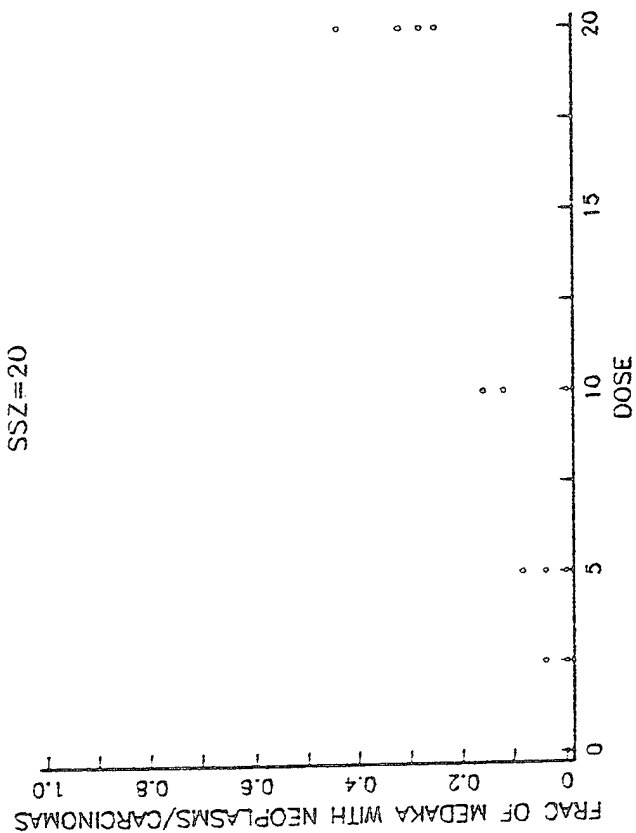
Incidences of Hepatocellular Neoplasms (Adenoma[s] and/or Carcinoma[s]) Combined. Numerator is Number of Fish with Combined Hepatocellular Neoplasms. Denominator is Number of Fish (Livers) Examined.

Time Post Initial Exposure	Fish at 6 Days of Age at Start of Test																			
	Control Groups				2.5 mg/L Groups				5.0 mg/L Groups				10.0 mg/L Groups				20.0 mg/L Groups			
	13	21	35	54	09	18	48	49	16	17	42	62	32	41	58	14	11	25	33	60
4 Months	0/25	0/24	0/25	0/25	0/25	0/24	1/25	0/25	0/25	2/24	1/25	0/25	3/25	4/25	0/25	3/25	7/25	8/25	6/24	11/25
6 Months	0/25	0/24	0/25	0/25	2/25	6/25	1/25	0/25	1/25	3/25	6/25	6/24	5/25	11/25	7/25	13/25	21/25	16/25	19/25	18/25
9 Months	0/14	2/11	0/12	0/23	3/16	1/11	8/19	6/21	6/19	11/18	6/14	10/15	12/16	13/16	9/14	11/14	18/18	10/13	7/7	14/16

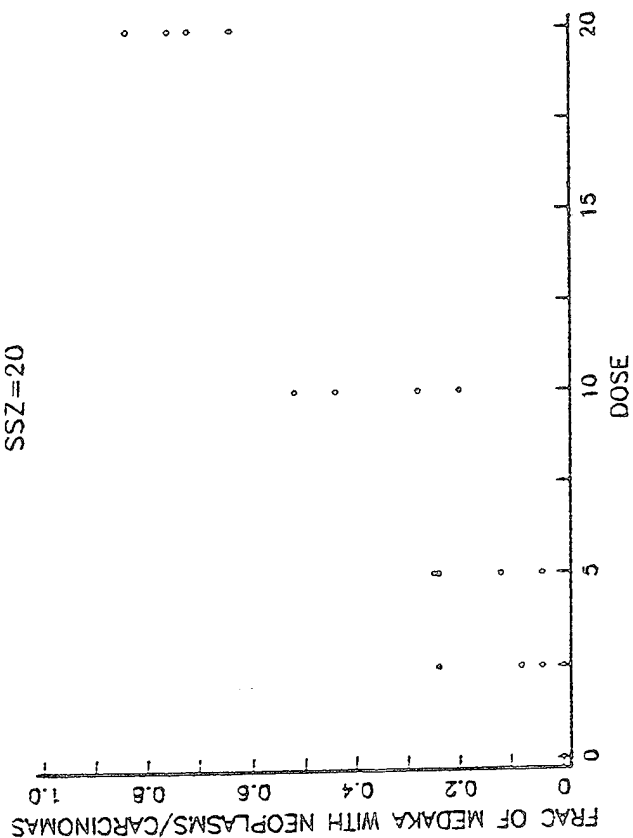
Time Post Initial Exposure	Fish at 52 Days of Age at Start of Test																			
	Control Groups				2.5 mg/L Groups				5.0 mg/L Groups				10.0 mg/L Groups				20.0 mg/L Groups			
	01	30	39	52	06	29	43	61	07	26	37	63	03	23	40	56	12	20	34	57
4 Months	0/25	1/25	0/24	1/24	1/25	0/25	0/24	0/25	0/25	0/25	0/25	0/24	0/24	1/24	1/25	1/24	0/25	2/25	4/24	2/25
6 Months	0/25	0/24	0/25	0/25	2/25	0/25	0/25	2/25	4/25	3/25	4/25	1/25	3/25	4/25	4/25	6/25	6/25	10/25	9/25	6/25
9 Months	1/24	0/22	0/24	1/24	7/22	1/24	3/23	1/19	4/23	3/17	3/23	1/23	7/21	3/21	10/23	6/22	10/17	15/23	11/20	12/19

FRAC OF MEDAKA WITH AT LEAST 1 NEOPLASM/CARCINOMA
AGE 8 DAYS AT START OF EXPERIMENT

,TIME=4
SSZ=20



,TIME=6
SSZ=20



,TIME=9
SSZ=20

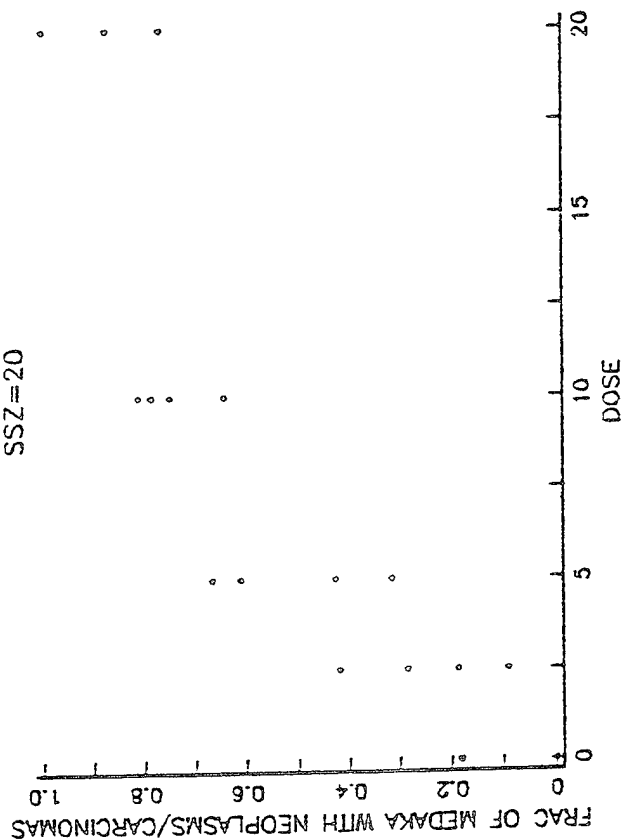


Figure 1

FRAC OF MEDAKA WITH AT LEAST 1 NEOPLASM/CARCINOMA
AGE 6 DAYS AT START OF EXPERIMENT

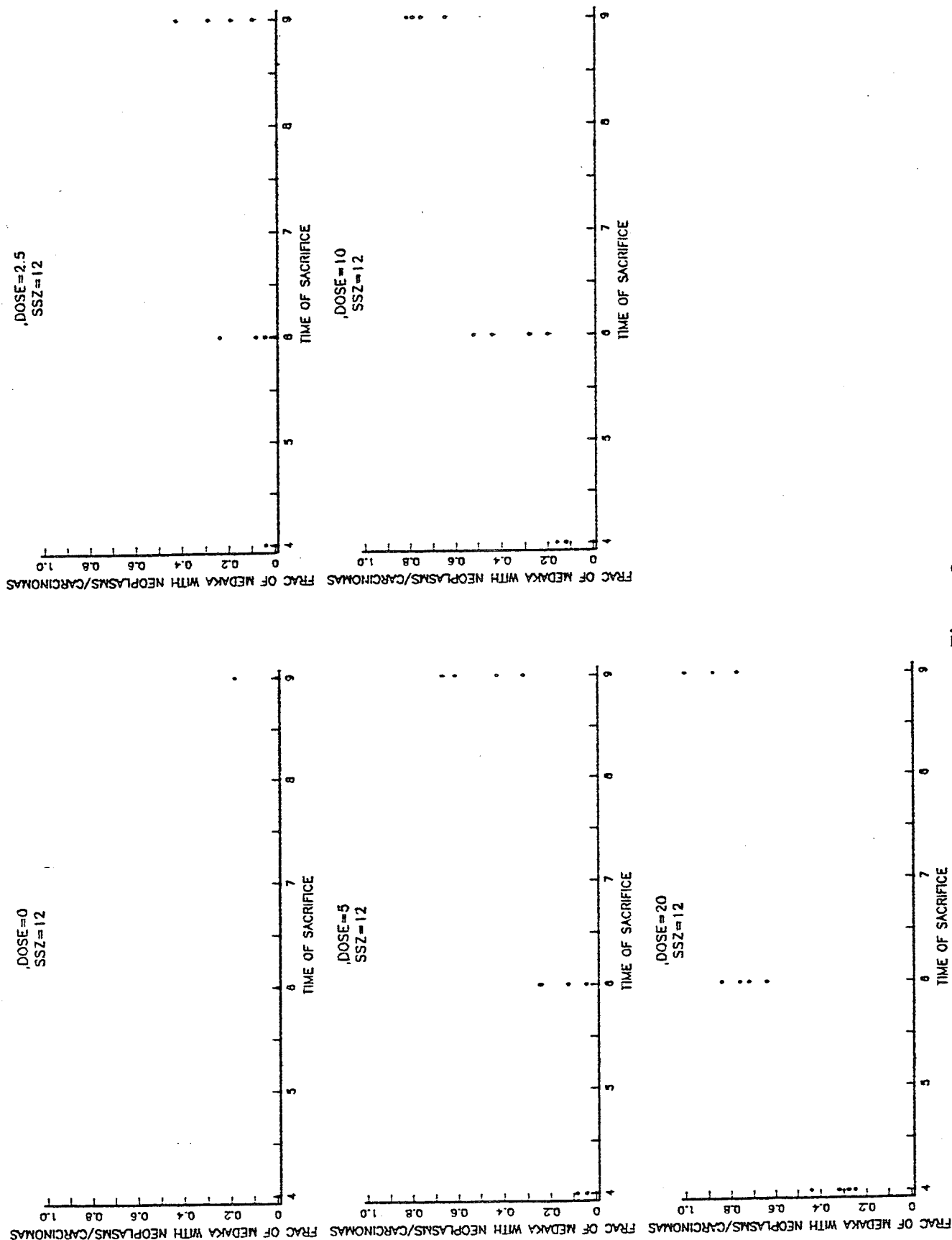
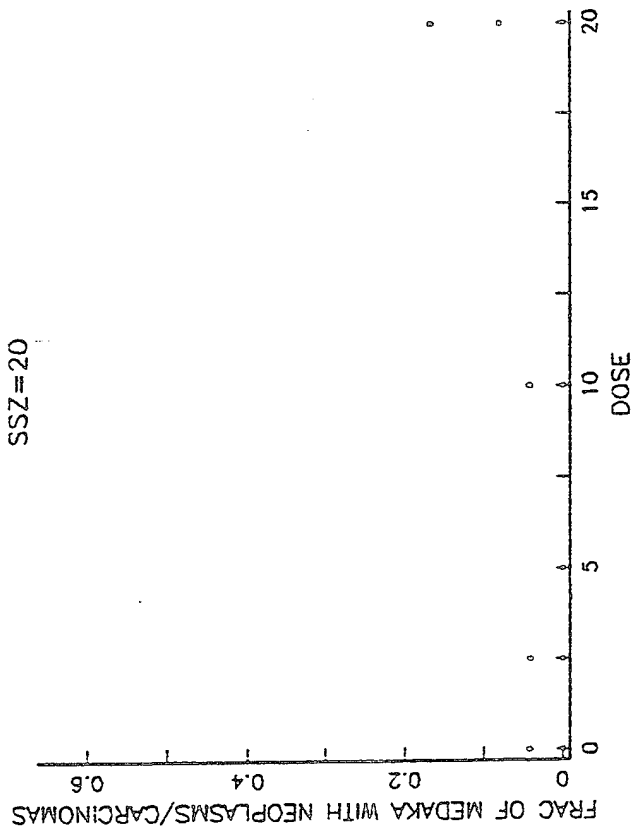


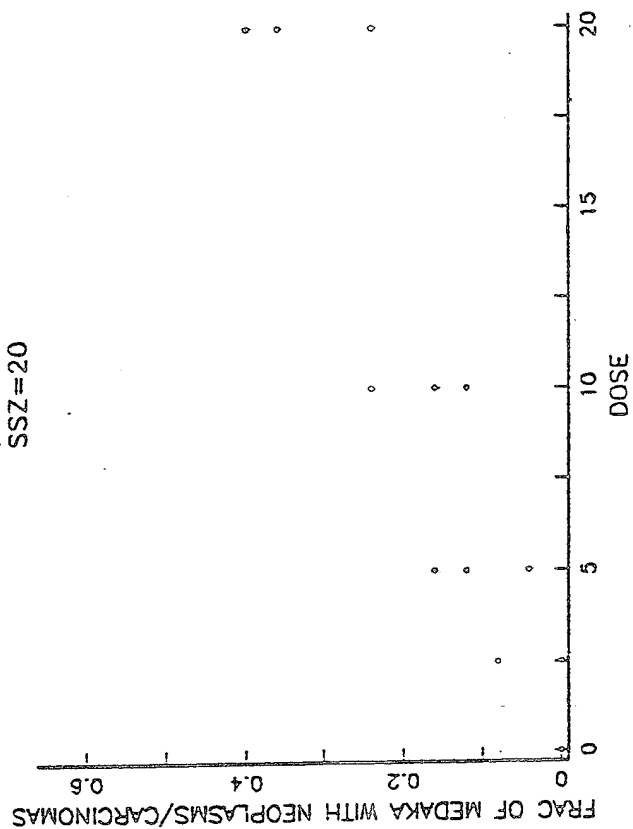
Figure 2

FRAC OF MEDAKA WITH AT LEAST 1 NEOPLASM/CARCINOMA
AGE 52 DAYS AT START OF EXPERIMENT

.TIME=4
SSZ=20



.TIME=6
SSZ=20



.TIME=9
SSZ=20

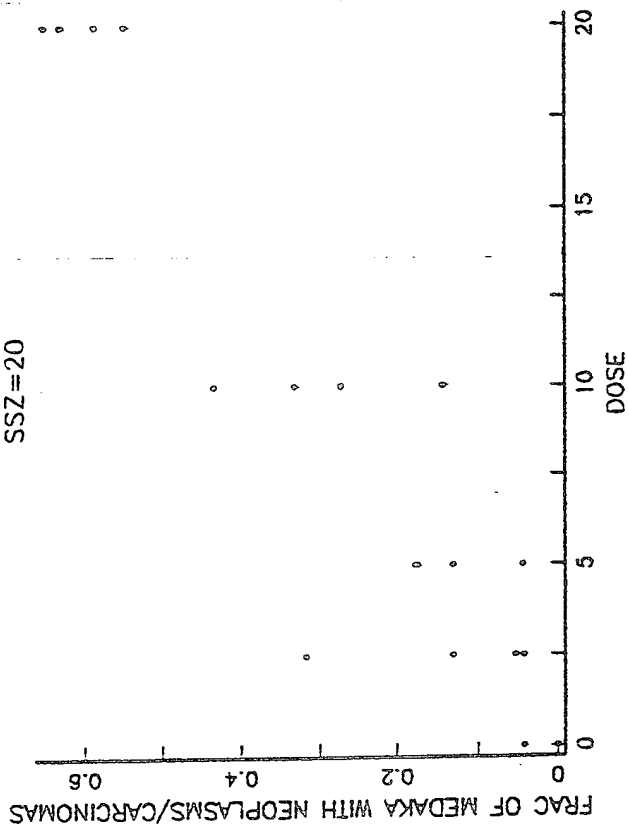


Figure 3

FRAC OF MEDAKA WITH AT LEAST 1 NEOPLASM/CARCINOMA
AGE 52 DAYS AT START OF EXPERIMENT

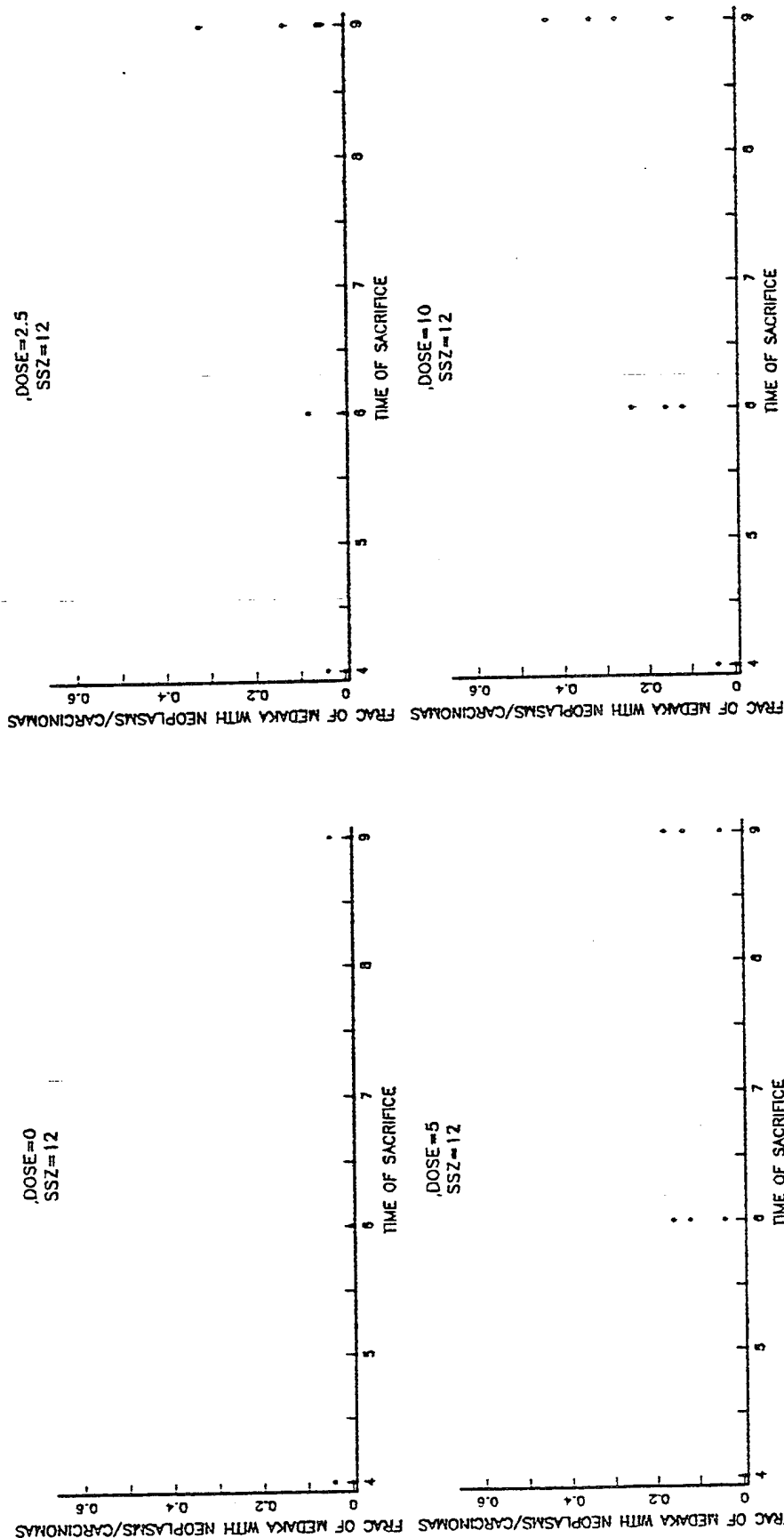


Figure 4

LOG ODDS OF NO NEOPLASMS/CARCINOMAS OCCURRING
MEDAKA OF AGE 6 DAYS AT START OF EXPERIMENT

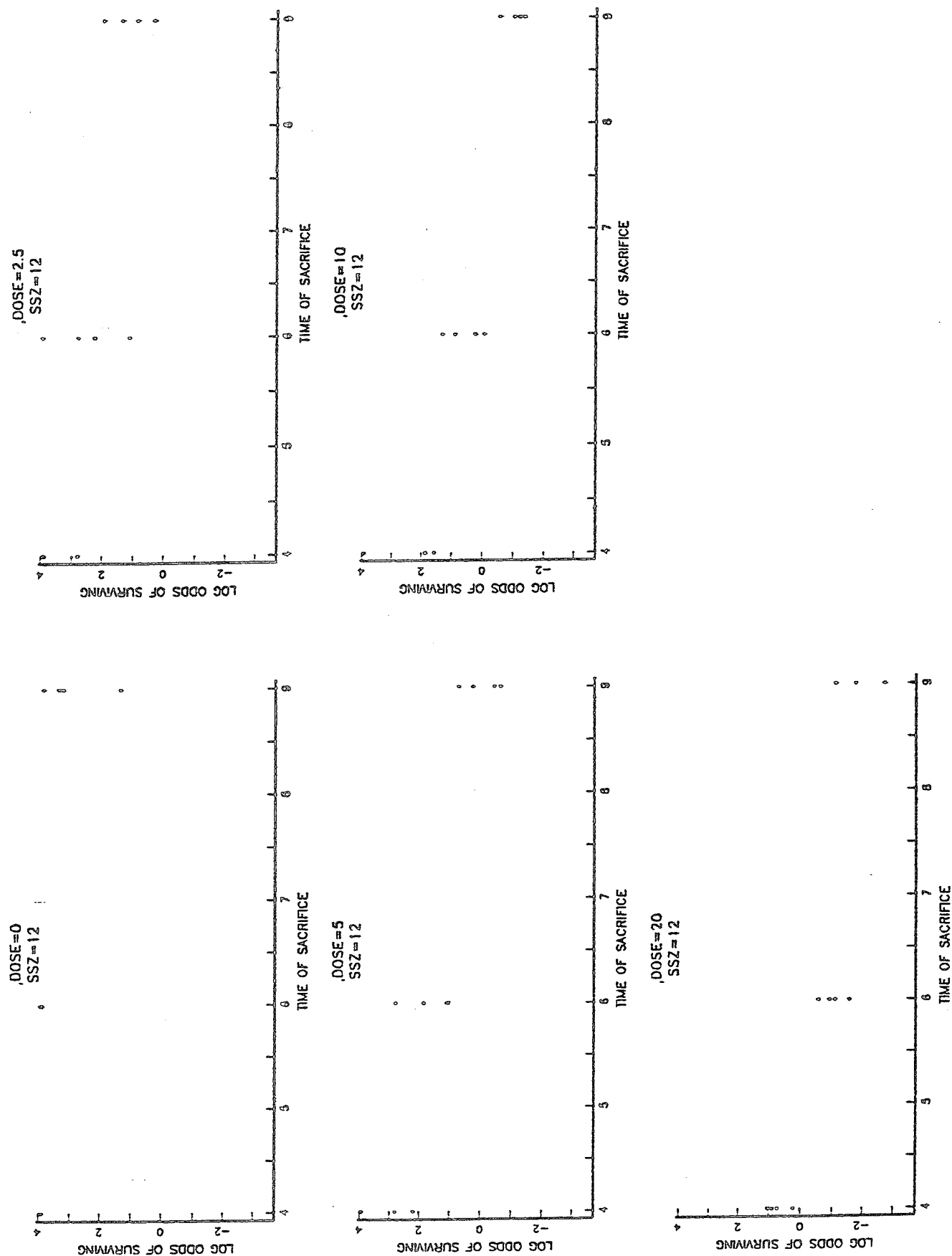
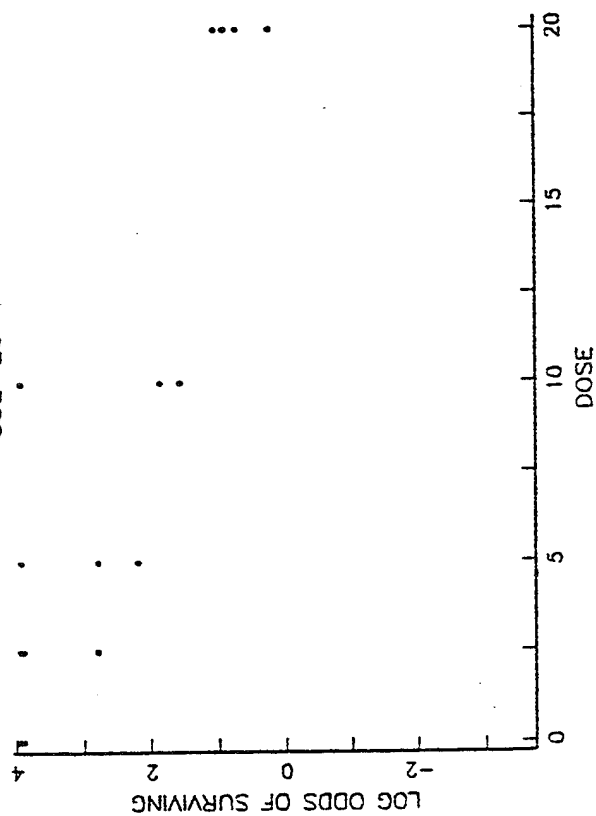


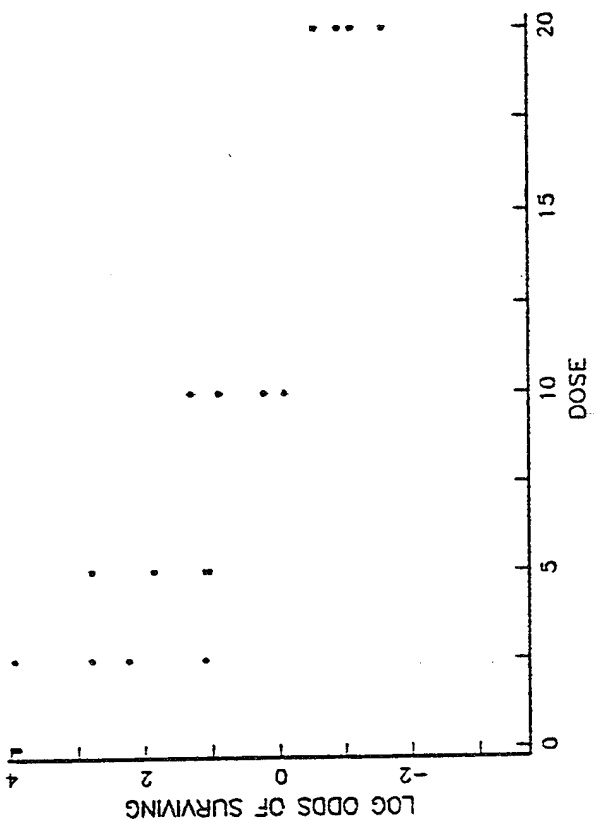
Figure 5

LOG ODDS OF NO NEOPLASMS/CARCINOMAS OCCURRING MEDAKA OF AGE 6 DAYS AT START OF EXPERIMENT

,TIME=4
SSZ=20



,TIME=6
SSZ=20



,TIME=9
SSZ=20

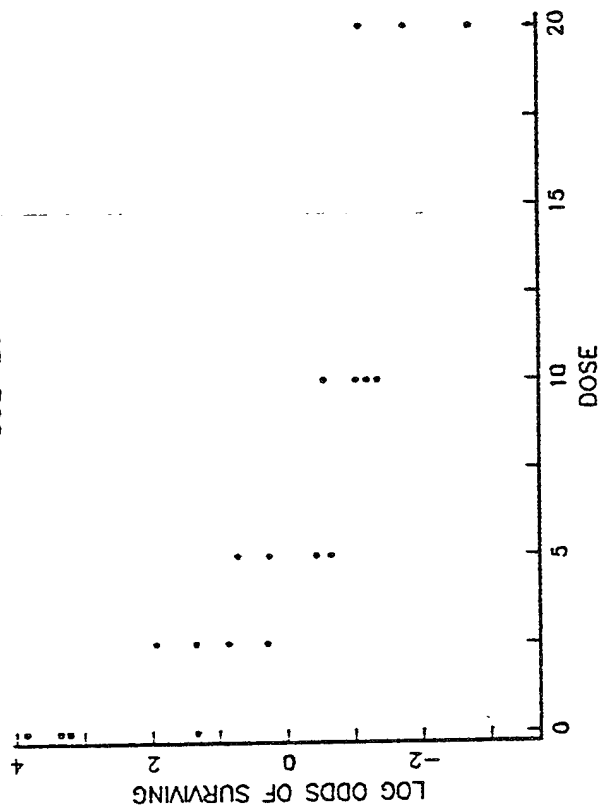


Figure 6

LOG ODDS OF NO NEOPLASMS/CARCINOMAS OCCURRING
MEDAKA OF AGE 52 DAYS AT START OF EXPERIMENT

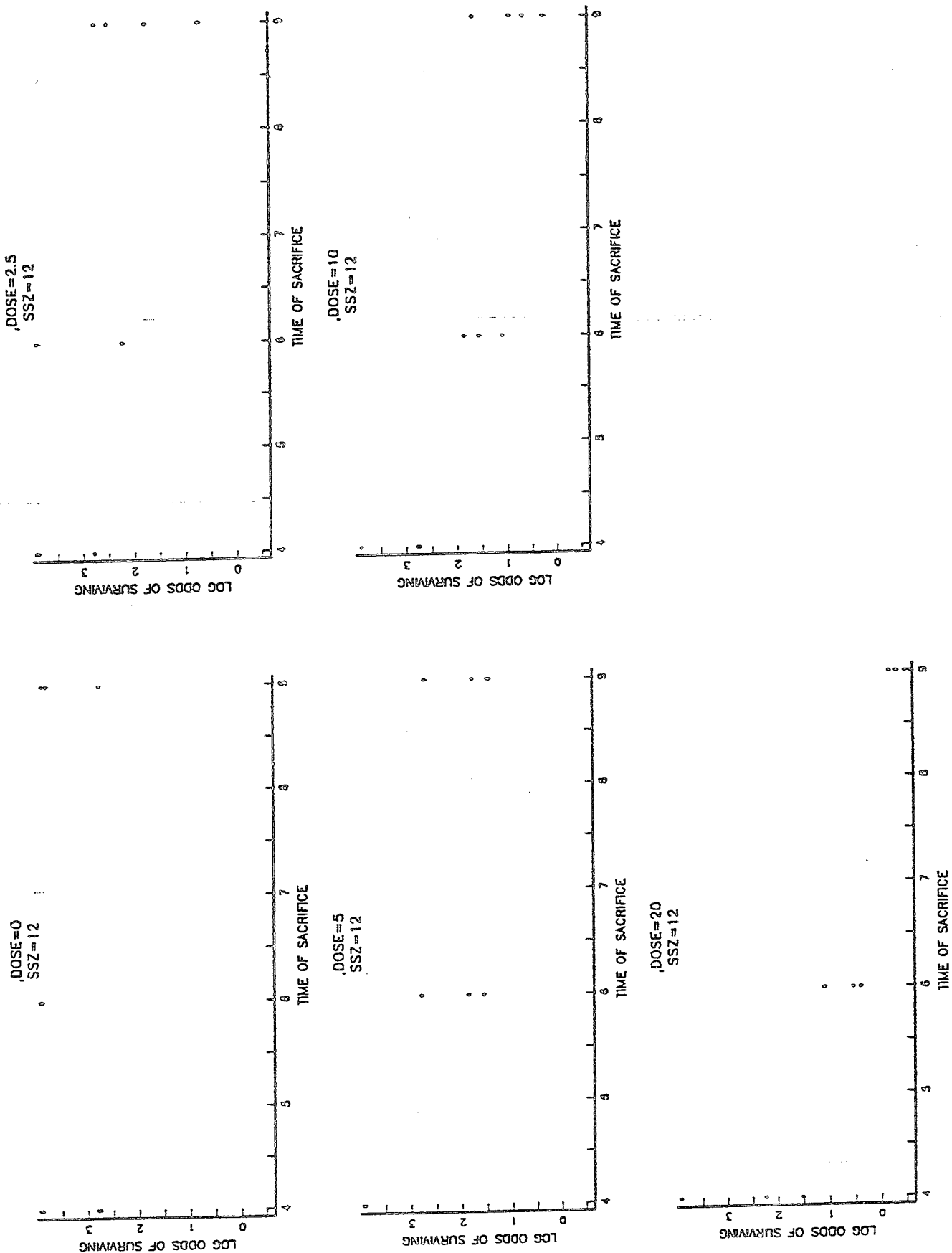
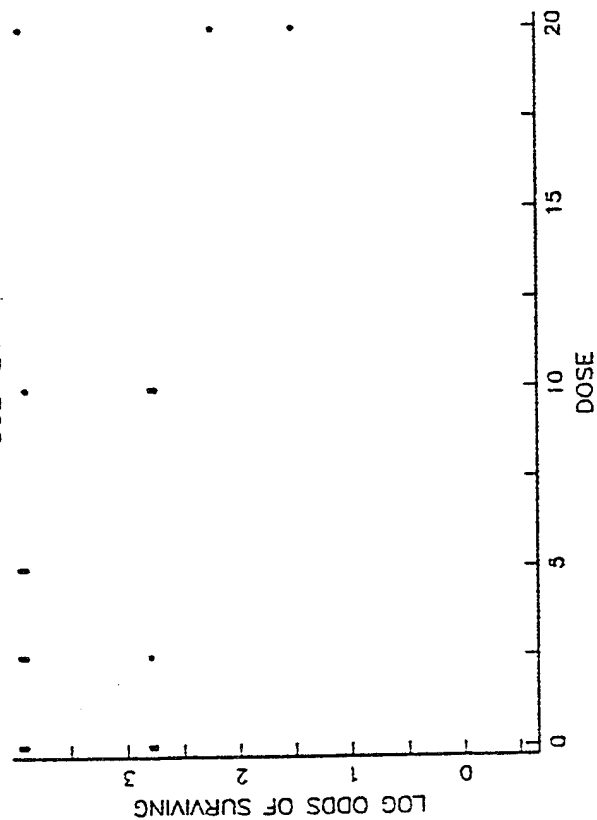


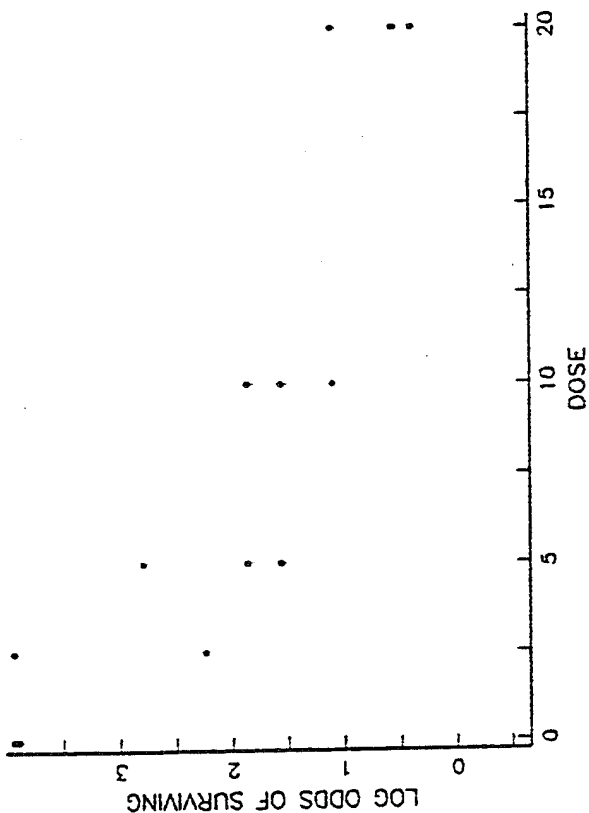
Figure 7

LOG ODDS OF NO NEOPLASMS/CARCINOMAS OCCURRING
MEDAKA OF AGE 52 DAYS AT START OF EXPERIMENT

,TIME=4
SSZ=20



,TIME=6
SSZ=20



,TIME=9
SSZ=20

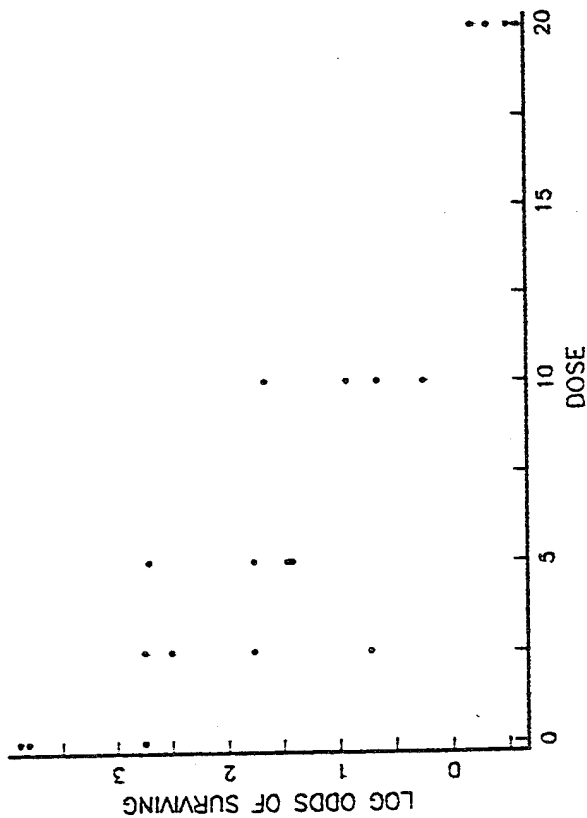


Figure 8

APPENDIX 2

ASSESSMENT OF LIVER MODIFICATION AND CELL PROLIFERATION IN MEDAKA UNDER DEN AND TCE CONCENTRATION: USING DATA AVAILABLE 4/26/94 Suggestions for Statistical Analysis and Results from Exploratory Data Analysis

D. P. Gaver

P. A. Jacobs

Some Questions and Areas for Investigation

Here are some questions, areas of investigation, and statistical approaches to the *Medaka* data sets (Sacrifice B, and potentially Sacrifice D). We also describe results of some exploratory data analyses performed at the Naval Postgraduate School.

1. Data and Data Summaries

(a) There are missing data, e.g. fish 58, 67. There may be others. These are coded 99999. Leave these out; do not enter the above defaults!

(b) There are some obvious outliers, e.g. fish 16's weight, which is surprisingly low. These can distort automatic fits or ANOVAs. If spotted they can be removed. We should investigate outlier causes. Possibly the obvious outliers should be revisited, images redone, also summary numbers.

(c) Not all fish have 5 slices examined.

If *robust statistical methods* were used (unresponsive to aberrant values) both (a) and (b) could be accomplished automatically: the outliers would be de-emphasized. Eventually we could go in that direction. But it would be better to understand why they occurred and try to fix the situation.

2. Liver Area and Animal Weight

2.1. Some Suggestions for Statistical Analysis

(a) It is anticipated that fish liver area should be roughly positively correlated/associated with fish weight.

(b) Since dimensionally $AREA = (VOLUME)^{2/3}$, and hence for constant weight density

$$AREA = (WEIGHT)^{2/3},$$

it is worthwhile to examine plots that show both AREA vs. WEIGHT and AREA vs. $(WEIGHT)^{2/3}$.

In (c-1) and (c-2) below we describe results of exploratory data analysis of AREA vs. WEIGHT. The analysis is done on normalized residuals of area and normalized residuals of weight. We compute the normalized residuals as follows.

(b-1) Compute mean of all weights, mean of all $(weights)^{2/3}$, and mean of all areas. Compute standard deviation (\sqrt{Var}) of all weights, $(weights)^{2/3}$, and areas. Compute the normalized residuals for the i^{th} fish ($i = 1, 2, \dots, all$).

$$w_i = \frac{\text{weight } i^{th} \text{ fish} - \text{mean}(\text{weight})}{sd(\text{weight})}$$

$$w_{i(t=2/3)} = \left(\frac{(\text{weight } i^{th} \text{ fish})^{2/3} - \text{mean}(\text{weight})^{2/3}}{sd(\text{weight})^{2/3}} \right)$$

$$a_i = \frac{(\ell.\text{area } i^{th} \text{ fish}) - \text{mean}(\ell.\text{areas})}{sd(\ell.\text{areas})};$$

$\ell.\text{area}$ means *liver area*.

(b-2) Plot w_i vs. a_i

and $w_i(t=2/3)$ vs. a_i .

(b-3) Fit a straight-line regression to the residuals.

2.2. Results of Exploratory Data Analysis

(c-1) Table 1 displays the least squares regression estimates of residual liver area versus residual weight by treatment group. When we did this for controls we got a high coefficient of determination or R^2 , i.e. ~ 0.8 , which suggests a reasonable linear relation; plots show this too. Note the the R^2 -values are smaller for those treatment groups treated with DEN compared to those not treated with DEN for the same level of TCE.

(c-2) Figure 1 displays scatter plots of residual liver area versus residual weight for all data except 100 mg/ ℓ DEN as separate plots by treatment; the data from all tanks in a treatment group are combined; note that the plots are not on the same vertical axis. Figures 2-7 are plots of residual liver area versus residual weight for all tanks in each treatment group. Linear behavior of the above plots/regressions degenerates with concentration, particularly of DEN: the linearity is not at all apparent, and scatter around a fitted line becomes comparatively large; see right-hand side of Figure 1 and Figures 2-7. Note that the plot for the control group in Figure 1 (upper left-most) is on a different scale than other plots; one reason for this is the outlying value for fish 21. For some fish the liver areas grow greatly, while for others of nearly the same residual weight the residual is quite small.

Comment. The above effect of toxin on liver size *suggests* that response may not be a simple mean effect, but rather *may* tend to reflect the different effect of toxin concentration on different fish, i.e. *increase the variability of response*. This may mean that *variance*, rather than *mean* could be a useful quantifier of response to

nominal concentration. A partial explanation of the variability of liver response might be that nominal concentration and actual dose, at organ level, are very different. We know of no way to investigate this experimentally.

It seems possible that the above effect will be amplified by longer exposure. Examination of Sacrifice D data should be interesting.

TABLE 1
Linear Least Squares Regressions for
Residual Liver Area versus Residual Weight
Residual Liver Area = A + B (Residual Weight)
Estimates
[] = 95% normal confidence interval

TREATMENT		A	B	R ²	Variance of Regression Residual
DEN (mg/ℓ)	TCE (mg/ℓ)				
0 (control) (without fish 16)	0	0.19 [-0.06,0.44]	1.2 [0.79,1.5]	0.78	0.14
10	0	-0.39 [-0.83,0.06]	0.24 [-0.37,0.85]	0.05	0.58
0 (1 data point missing)	0.1	-0.43 [-0.61,-0.25]	0.56 [0.40,0.71]	0.82	0.10
10 (1 data point missing)	0.1	0.05 [-0.32,0.42]	0.49 [0.17,0.82]	0.45	0.40
0	1.0	0.13 [-0.27,0.53]	0.84 [0.40,1.3]	0.54	0.52
10	1.0	0.32 [-0.13,0.76]	0.41 [0.03,0.79]	0.28	0.50

3. Counting Index vs. Area Index

3.1. Some Suggestions for Statistical Analysis

(a) The counting index (CI) and area index (AI) both *tend* to measure the same quantity, namely the numbers of cells in S-phase within the ROI. An increase in that number is supposed to indicate cell proliferation related to the concentration, i.e. DEN and TCE in various concentrations.

(b) The area index may tend to grow (or otherwise change) over time and with concentration because of the effect of DEN and/or TCE on cell *size*, represented by presented (stained/black, also that of other cells) area in a slice. The number of stained cells may also grow, but the former effect, on AI, may be (i) greater, and (ii) make it more variable, as time and toxin level increase. There is the possibility of bias, and extra variability. It might actually be that the *number* of cells in S-phase stays the same or decreases with increased concentration, but the size/area of those remaining increases more than enough to overcome this.

(c) *Recommendation:*

(1) Study graphically (plot) AI vs CI for various concentration levels.

(2) Do so for all data combined in a treatment group, and by replicate; the latter should indicate between-tank variation.

This has been (partially) done at BRDL. The indication is of a fairly linear relation, but the variability increases with toxin concentration.

3.2. Results of an Exploratory Data Analysis

3.2.1. Preliminary Study of Area Index (AI) by Treatment

This subsection reports results of a preliminary look at area index

$$\text{area index} = \frac{\text{area of positive hepatocytes}}{\text{area of region of interest}}$$

(without multiplying by 100).

Figure 8 is a scatter plot of residual mean area of the region of interest (ROI) versus the residual mean area of the positive hepatocytes where

$$\begin{array}{l} \text{residual mean} \\ \text{area of the region} \\ \text{of interest} \end{array} = \frac{x_{ri\text{area}} - \text{Mean}(x_{ri\text{area}})}{\sqrt{\text{Variance}(x_{ri\text{area}})}}$$

and

$$\begin{array}{l} \text{residual mean area} \\ \text{of the positive} \\ \text{hepatocytes} \end{array} = \frac{(x_{hep\text{are}}) - \text{Mean}(x_{hep\text{are}})}{\sqrt{\text{Variance}(x_{hep\text{are}})}}$$

The scatter plot indicates that there is no relation between the mean area of the region of interest and the mean area of the positive hepatocytes. It also indicates that Fish 21 is an outlier.

Figure 9 displays box plots of the area index (mean area of positive hepatocytes = $x_{hep\text{are}}$) / (mean area of region of interest = $x_{ri\text{area}}$) by treatment group; the mean is over the slices; note that our area index is the ratio not multiplied by 100. The treatment group of 100 mg/ℓ of DEN was omitted. The upside of the box is at the 75% quantile of the area index. The lower side of the box is at the 25% quantile. The mean of the area index is represented as a circle in the box and the median is a line across the box. Fish 21 is omitted.

Figure 10 displays box plots of the mean area of positive hepatocytes by the same treatment group. Fish 21 is omitted.

Figures 9 and 10 are not that much different. Both plots indicate the major difference in response is between the control group and the DEN with 1.0 mg/ℓ group.

Box plots are summary plots. To further investigate possible associations of area index and treatment, the area indices are plotted against treatments. Figure 11 presents the area indices versus the level of TCE for all data. The levels of TCE are those found on the tables. In particular, fish 81–96 are exposed to

0 mg/ℓ DEN and 1.425 mg/ℓ TCE and fish 97–112 are exposed to 10.0 mg/ℓ DEN and 1.339 mg/ℓ level of TCE. There does not appear to be much association.

Figure 12 plots the area index versus level of DEN exposure. Again, no association is apparent.

Figure 13 displays the area indices of fish not exposed to DEN versus TCE exposure. Again, no strong association is apparent.

Figure 14 displays plots of area indices for fish exposed to DEN versus TCE exposure. There seems to be some association. The area index for fish 105 appears lower than those of the other fish in the 10 mg/ℓ DEN and 1.4 mg/ℓ TCE group.

3.2.2. Preliminary Study of Associations Between the Count Index and Area Index

In this subsection we investigate associations between the count index and area index by treatment group.

Figure 15 displays scatter plots of the count index versus the area index by treatment group. Table 2 displays the estimated parameters of the least squares regressions by treatment group.

$$\frac{\text{Mean Area of positive hepatocytes}}{\text{Mean Area of Region of Interest}} = A + B \frac{\text{Count of positive hepatocytes}}{\text{Total Cells Counted}}$$

where (count of positive hepatocytes = smposnuc) and (Total Cells Counted = tot_cell). Note that the estimates of A and B remain statistically the same across treatments. All coefficients of determination (R^2) are high (at least 0.7, often higher) *except* for the data pertaining to DEN = 10, TCE = 0.1, which gives $R^2 = 0.42$; this difference is also reflected in the scatter plot for that treatment which exhibits more variability than the other plots. There may be an explanation based on experimental aberrations. Maybe the data should be re-examined.

Figures 16–21 display the scatter plots by treatments with the linear least squares line shown. There is no strong evidence that the association between the count index and area index changes by treatment.

TABLE 2
Least Squares Regressions
Area Index = A + B (Count Index)
[] = 95% Confidence Interval

TREATMENT		A	B	R ²
DEN	TCE			
0	0	0 [-0.003,0.003]	0.31 [0.27,0.36]	0.98
10	0	0 [-0.13,.012]	0.31 [0.14,0.48]	0.76
0	0.1	0.003 [-0.009,0.015]	0.33 [0.18,0.48]	0.82
10 (Missing data point)	0.1	0.003 [-0.02,0.03]	0.33 [-0.12,0.79]	0.42
0	1.0	0.002 [-0.001,0.004]	0.42 [0.37,0.47]	0.99
10	1.0	0.002 [-0.004,0.008]	0.42 [0.34,0.50]	0.97

Figure 22 displays box plots of (count of positive hepatocytes)/(total cells counted) by treatment group. Figure 23 displays box plots of (mean area of positive hepatocytes)/(mean area of region of interest) by treatment group for those fish for which cells were also counted. Figure 24 (respectively Figure 26) displays (count of positive hepatocytes)/(total cells counted) by TCE level for 0 mg/ℓ DEN (respectively 10 mg/ℓ DEN). Figure 25 (respectively Figure 27) displays (mean area of positive hepatocytes)/(mean area of region of interest) by TCE level for 0 mg/ℓ DEN (respectively 10 mg/ℓ DEN) for those fish for which cells were counted. The figures show little association with treatment.

3.2.3. Preliminary Study of Partial Data From Sacrifice D

In this section we describe results of a preliminary data analysis of the data from Sacrifice D. The data considered are the control treatment group and the (10 mg/ℓ DEN, 0 mg/ℓ TCE) treatment group. Both of these treatment groups have 1 missing observation.

Figure 28 displays a scatter plot and least squares straight line for residual liver area versus residual weight for the control group. Figure 29 is a similar display for the (10 mg/ℓ DEN, 0 mg/ℓ TCE) group. Table 3 displays the parameter estimates for the least squares lines. Since the 95% confidence intervals for the parameter estimates overlap the two regression lines are statistically the same and there is not that much difference in residual standard deviations.

Figure 30 displays $\frac{\text{Mean Area of Positive Hepatocytes}}{\text{Mean Area of the Region of Interest}}$ by level of DEN. The treatment with 10 mg/ℓ DEN is associated with higher and more variable ratios.

TABLE 3
Two Treatments from Sacrifice D
Least Squares Regression Estimates of the Linear Relation
Residual Liver Area = A + B (Residual Weight)

TREATMENT	ESTIMATES (standard errors) [95% confidence intervals]			
	A	B	Residual std. dev.	R ²
control	0 (0.08) [-0.17, 0.17]	0.95 (0.08) [0.78, 1.13]	0.31	0.91
10 mg/L DEN 0 mg/L TCE	0 (0.11) [-0.24, 0.24]	0.91 (0.11) [0.67, 1.16]	0.42	0.83

REFERENCE

IBM Corporation. A Graphical Statistical System (AGSS)

SCATTER PLOTS, SSZ=94, []=RANGE OF CONDITIONING VAR.

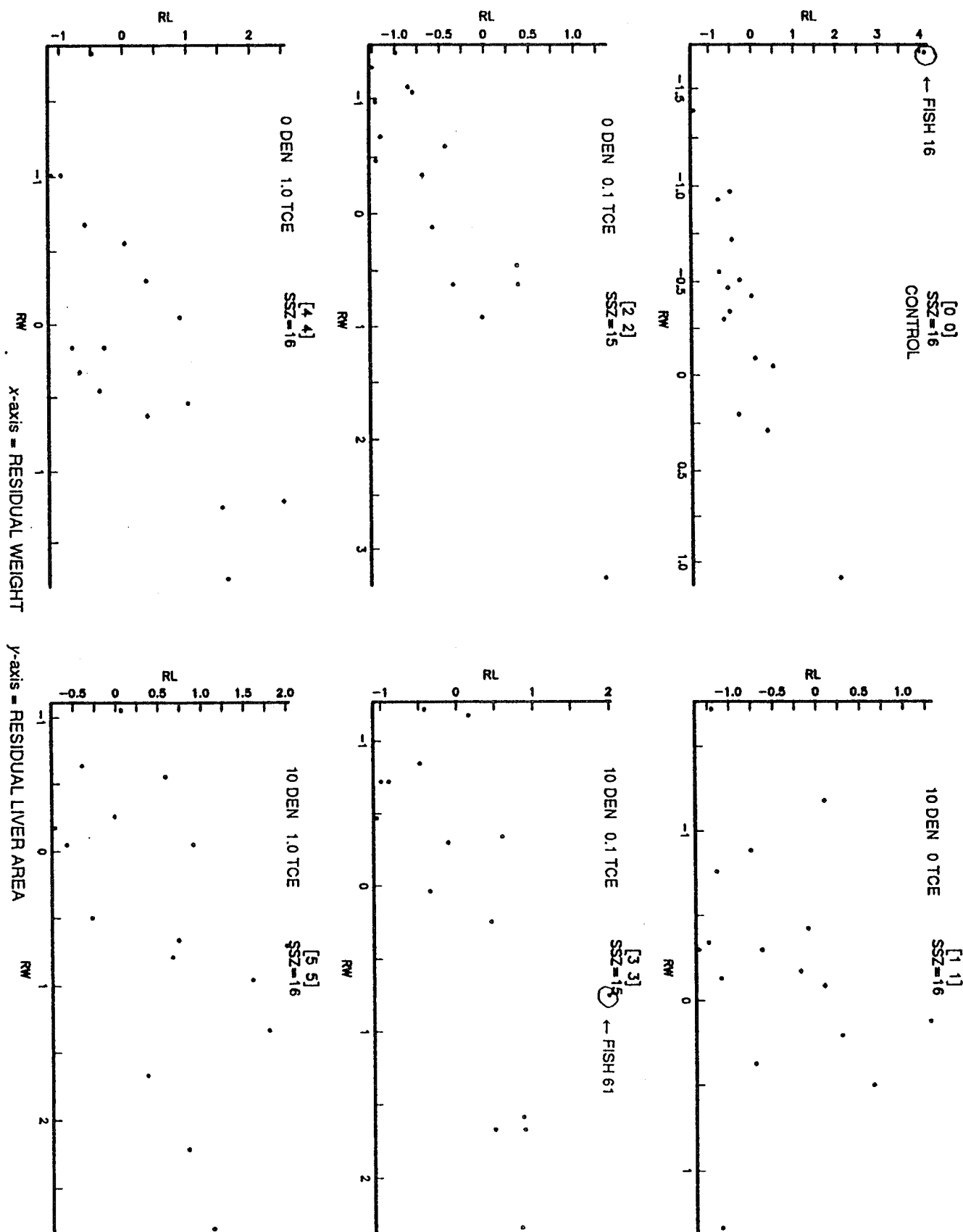


Figure 1

CONTROL WITH OUTLYING FISH 16 OMITTED

LS FIT: RES LIVER AREA = A + (B * RES WEIGHT)

A = .19; B = 1.16

95 PERC CI: A: [-.06 .44]; B: [.8, 1.5]

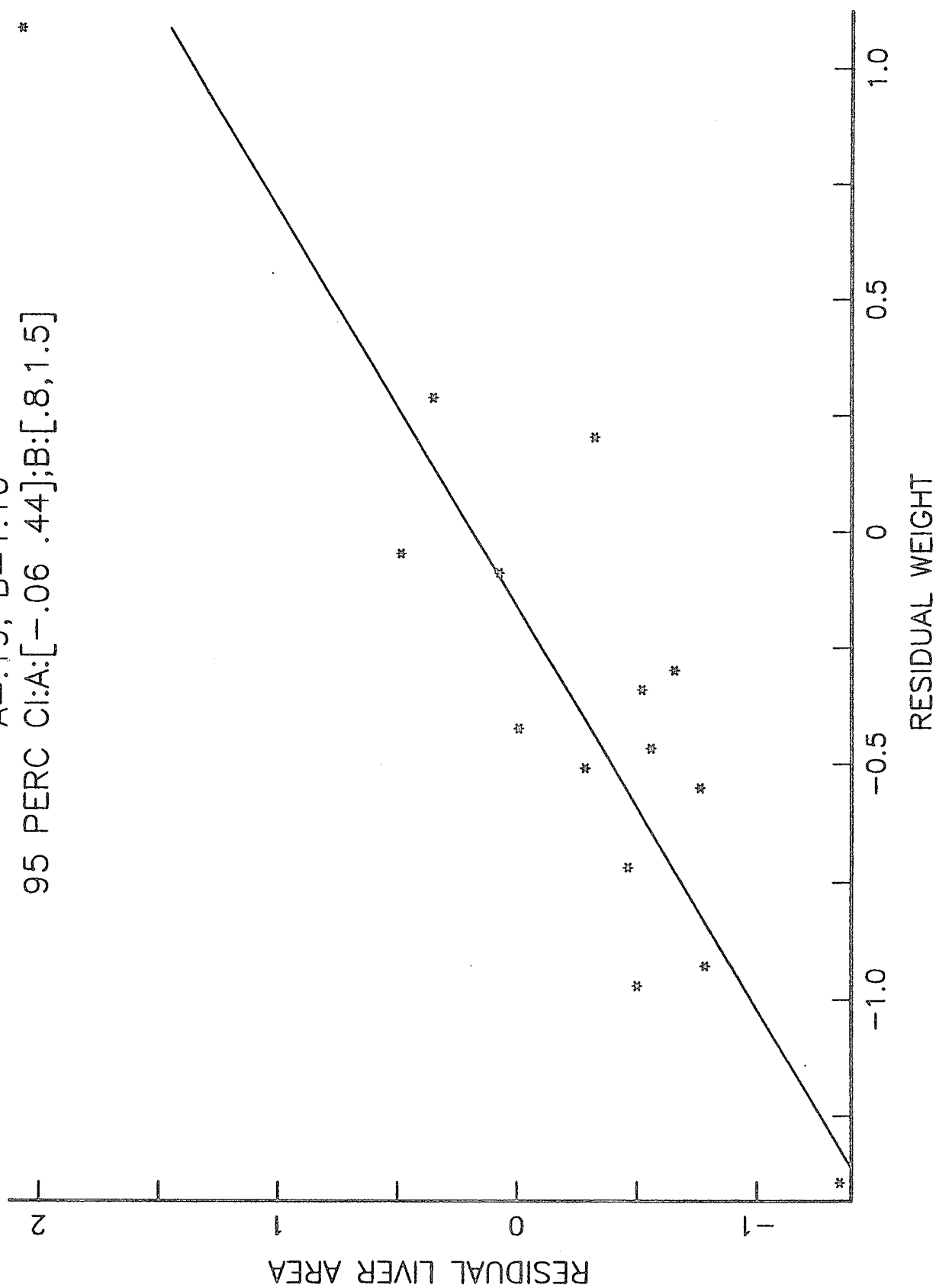


Figure 2

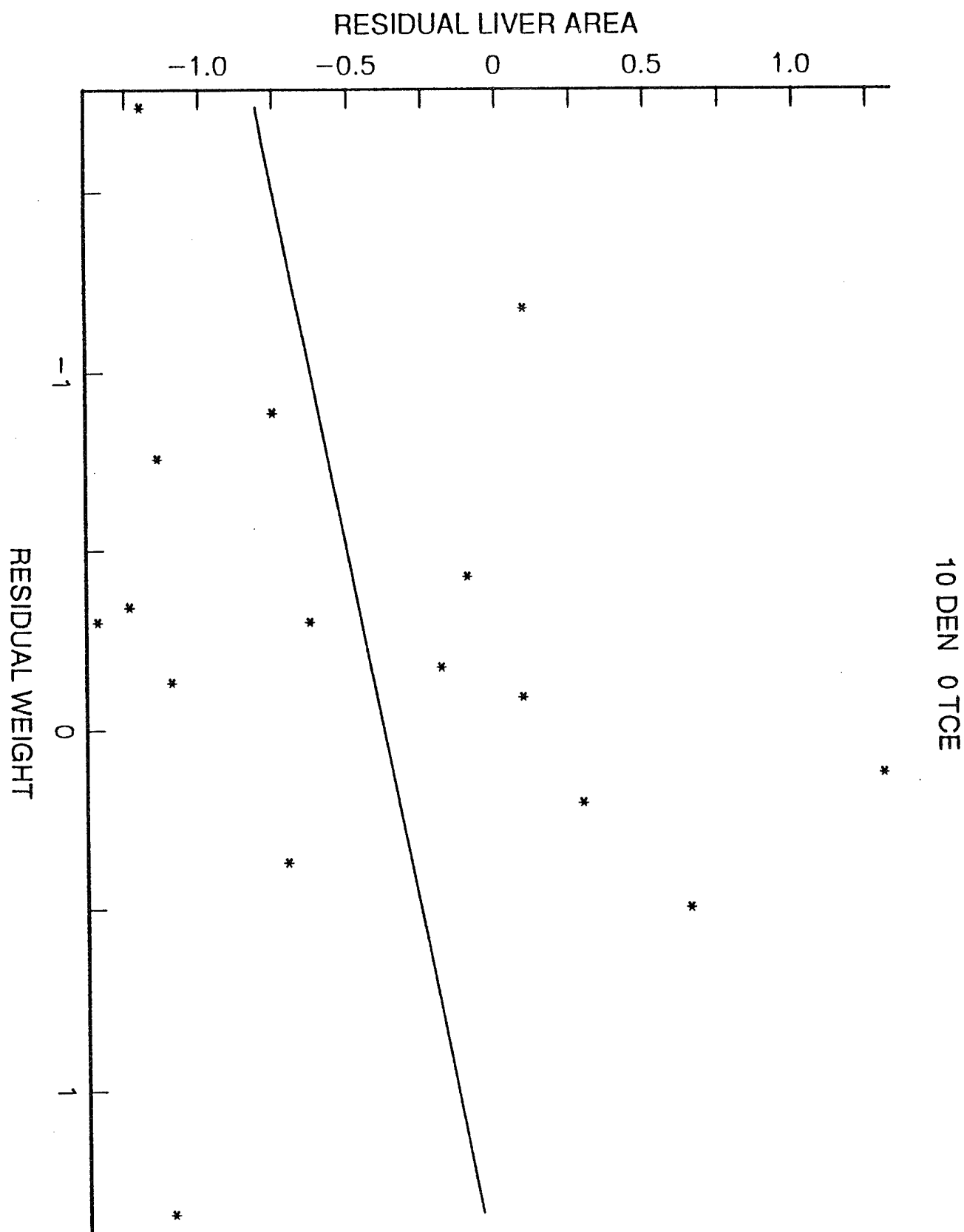


Figure 3

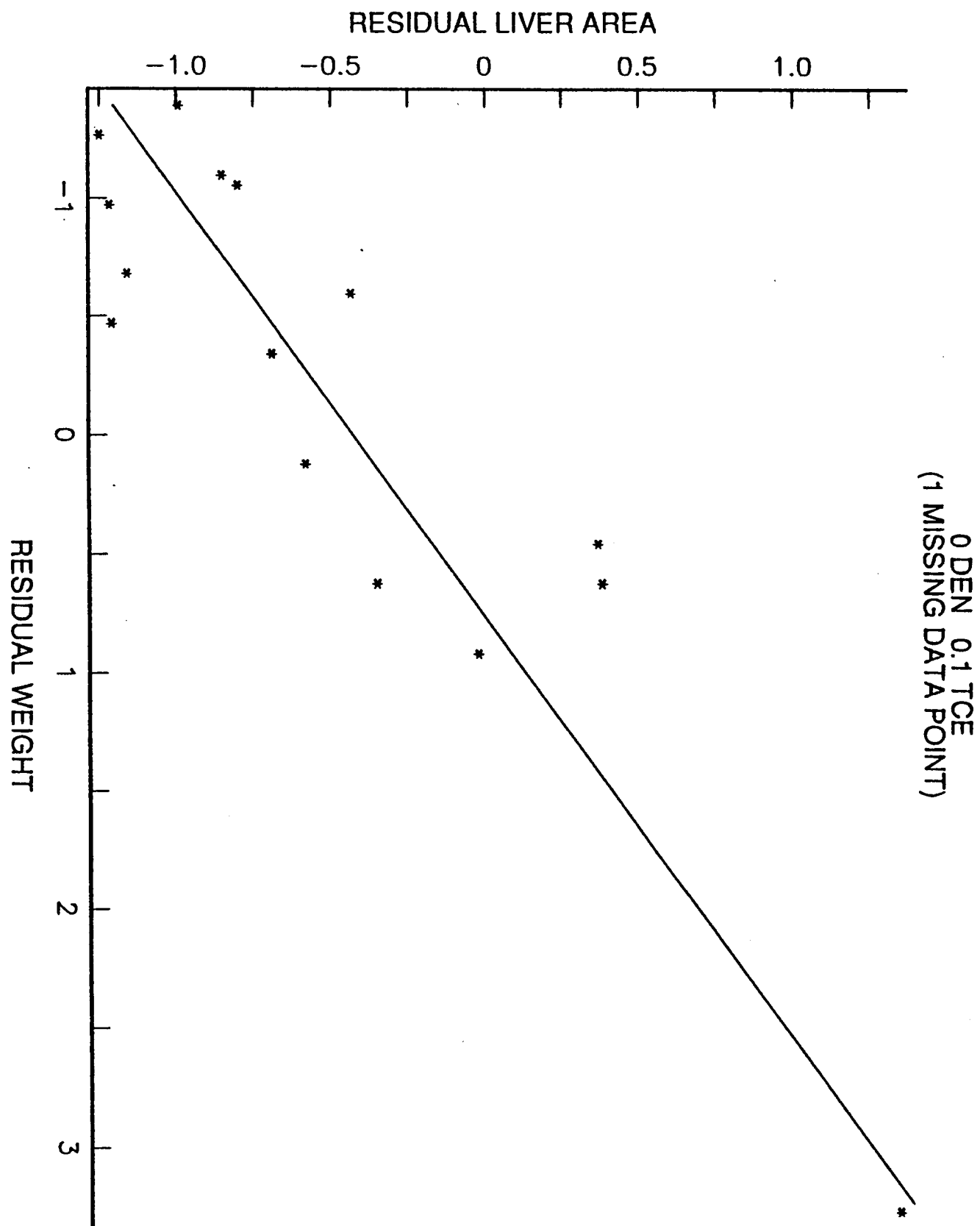


Figure 4

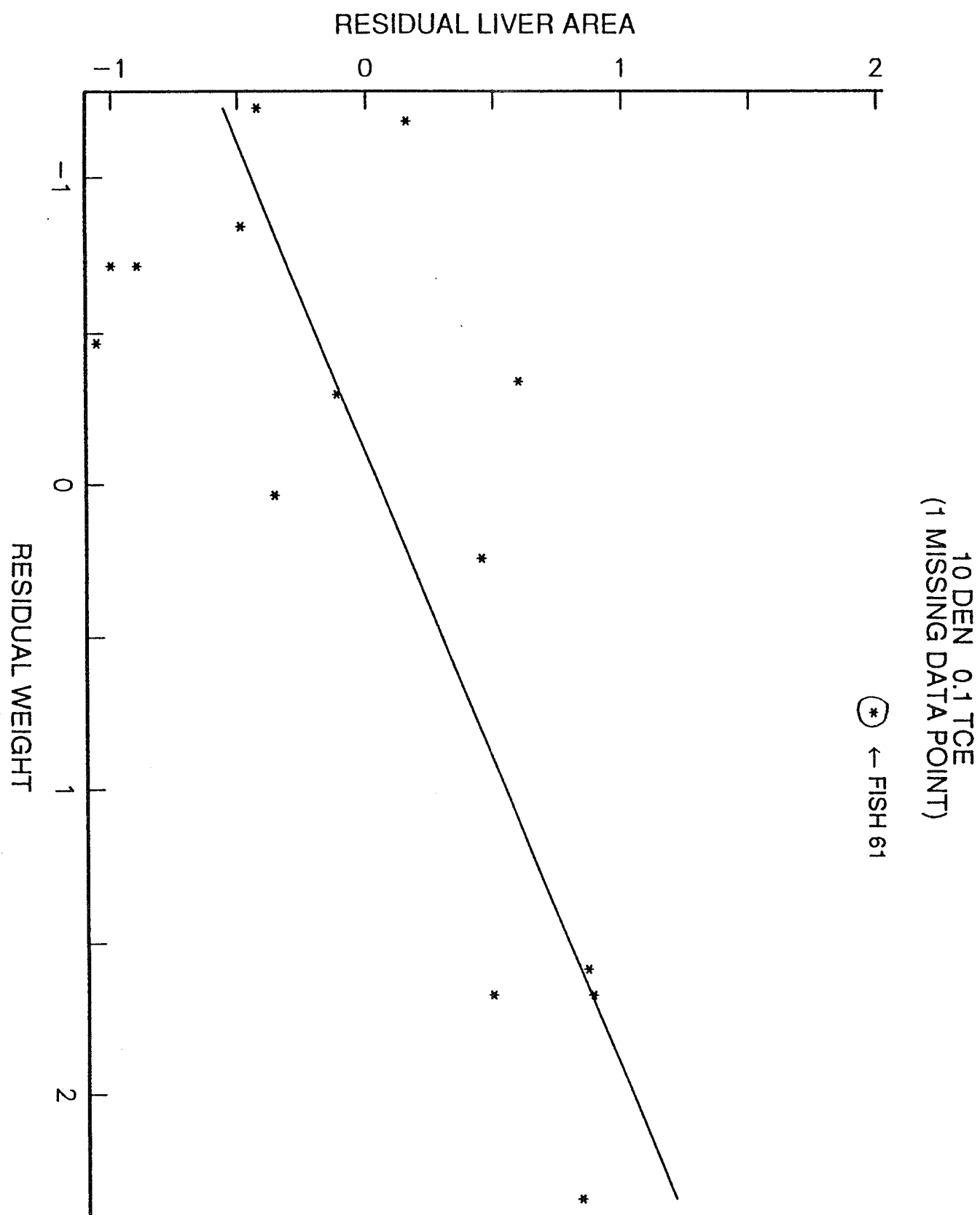


Figure 5

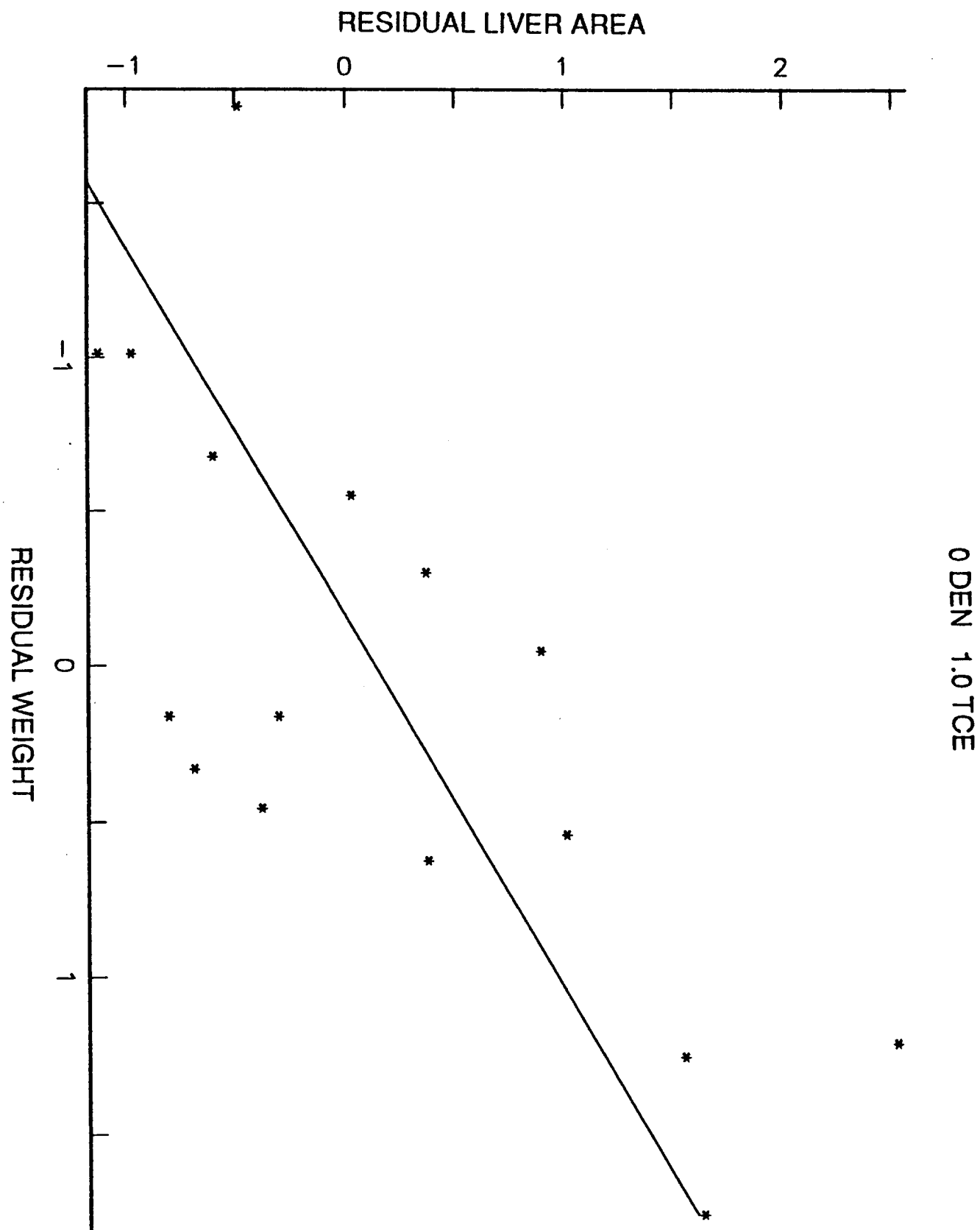


Figure 6

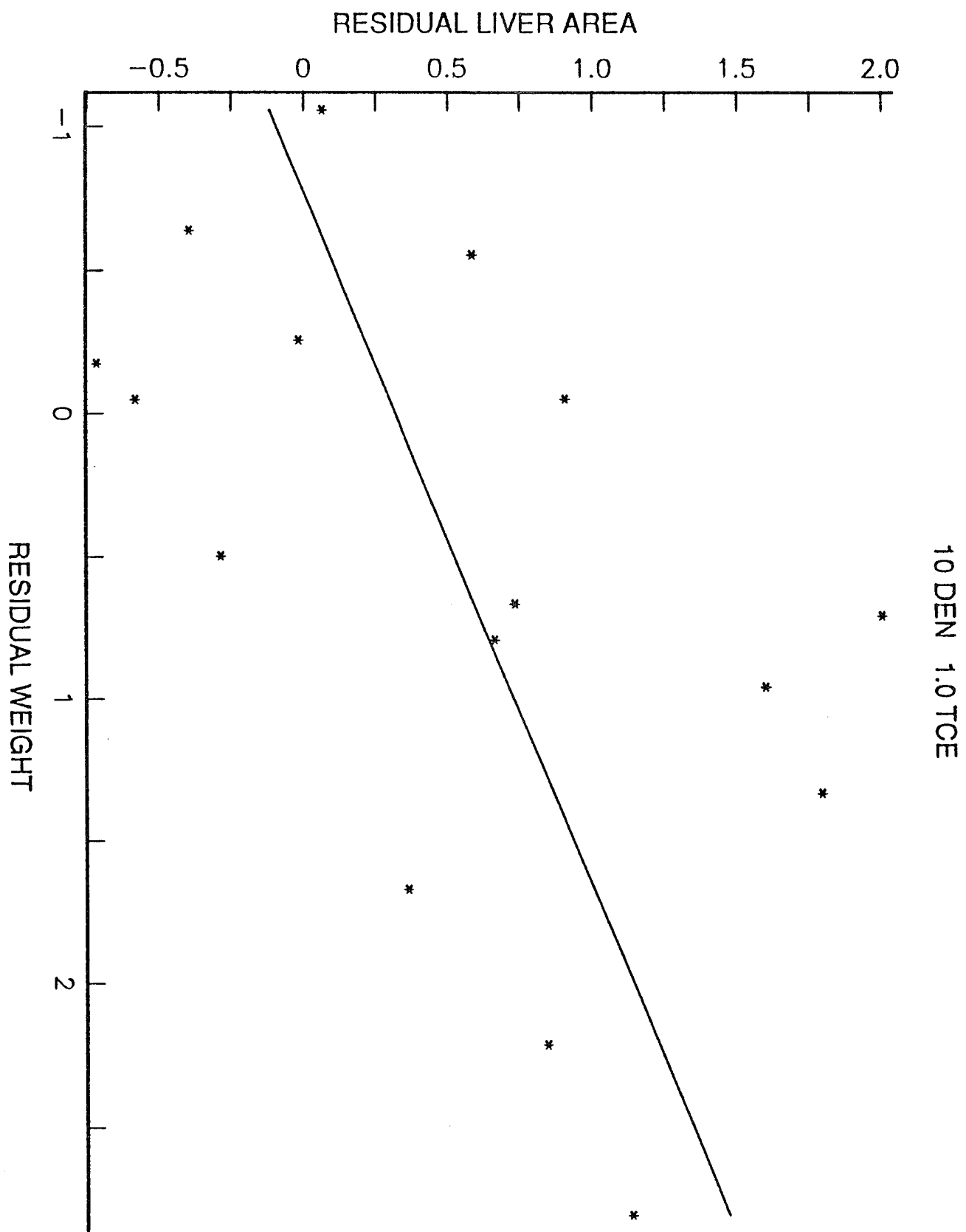


Figure 7

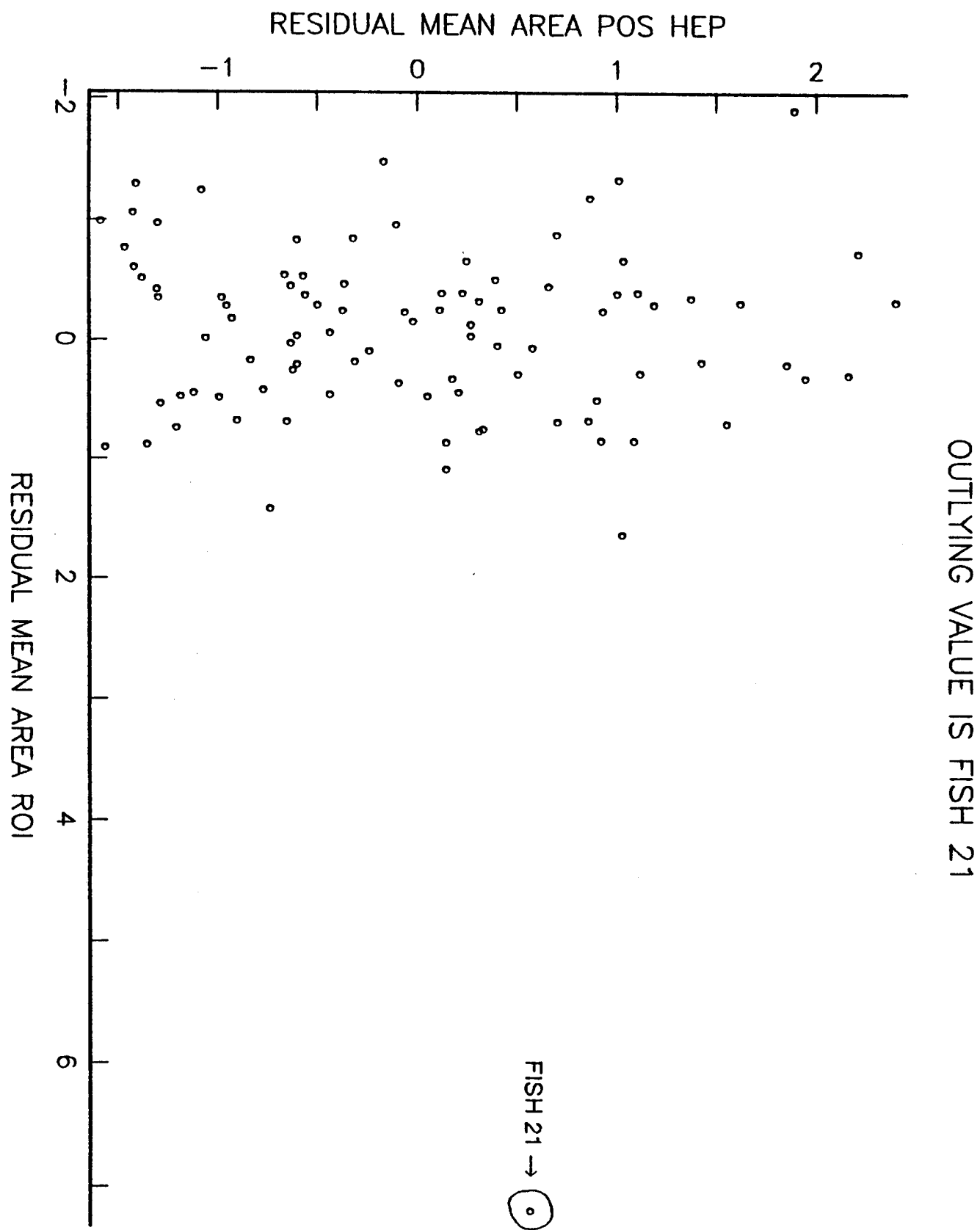


Figure 8

STRIP BOX/PERCENTILE PLOT, SSZ=93
without Fish 21

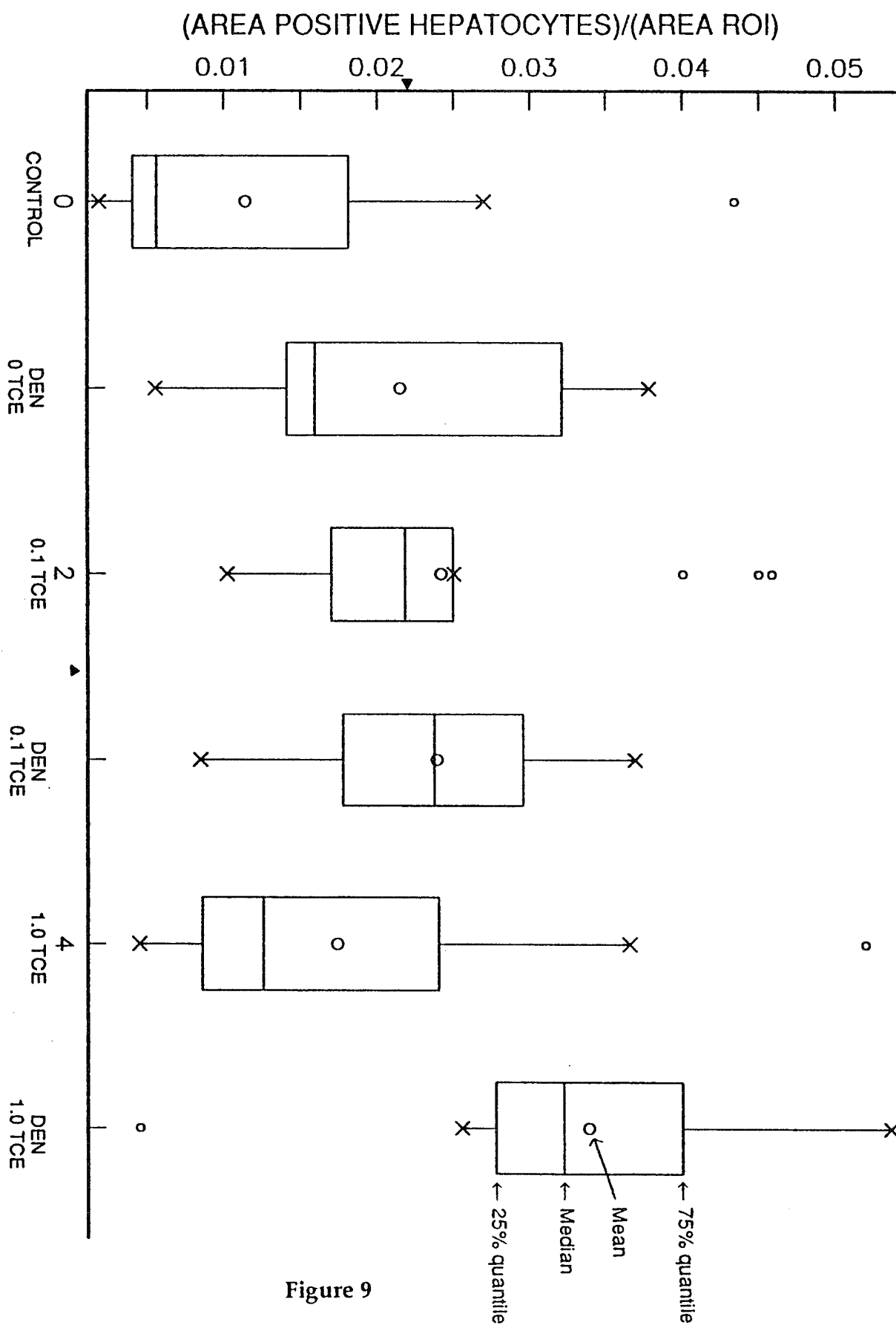


Figure 9

STRIP BOX/PERCENTILE PLOT, SSZ=93
without Fish 21

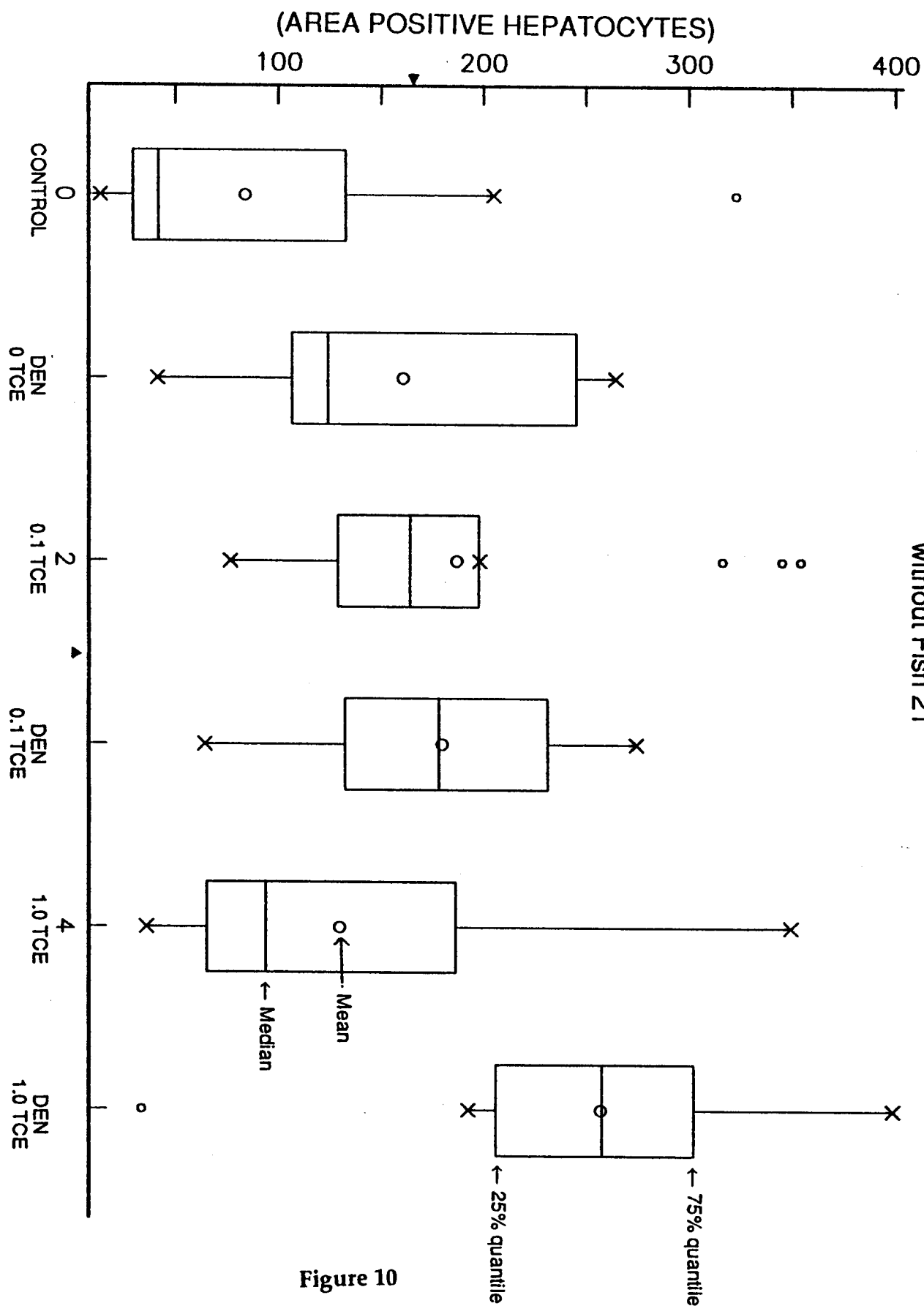


Figure 10

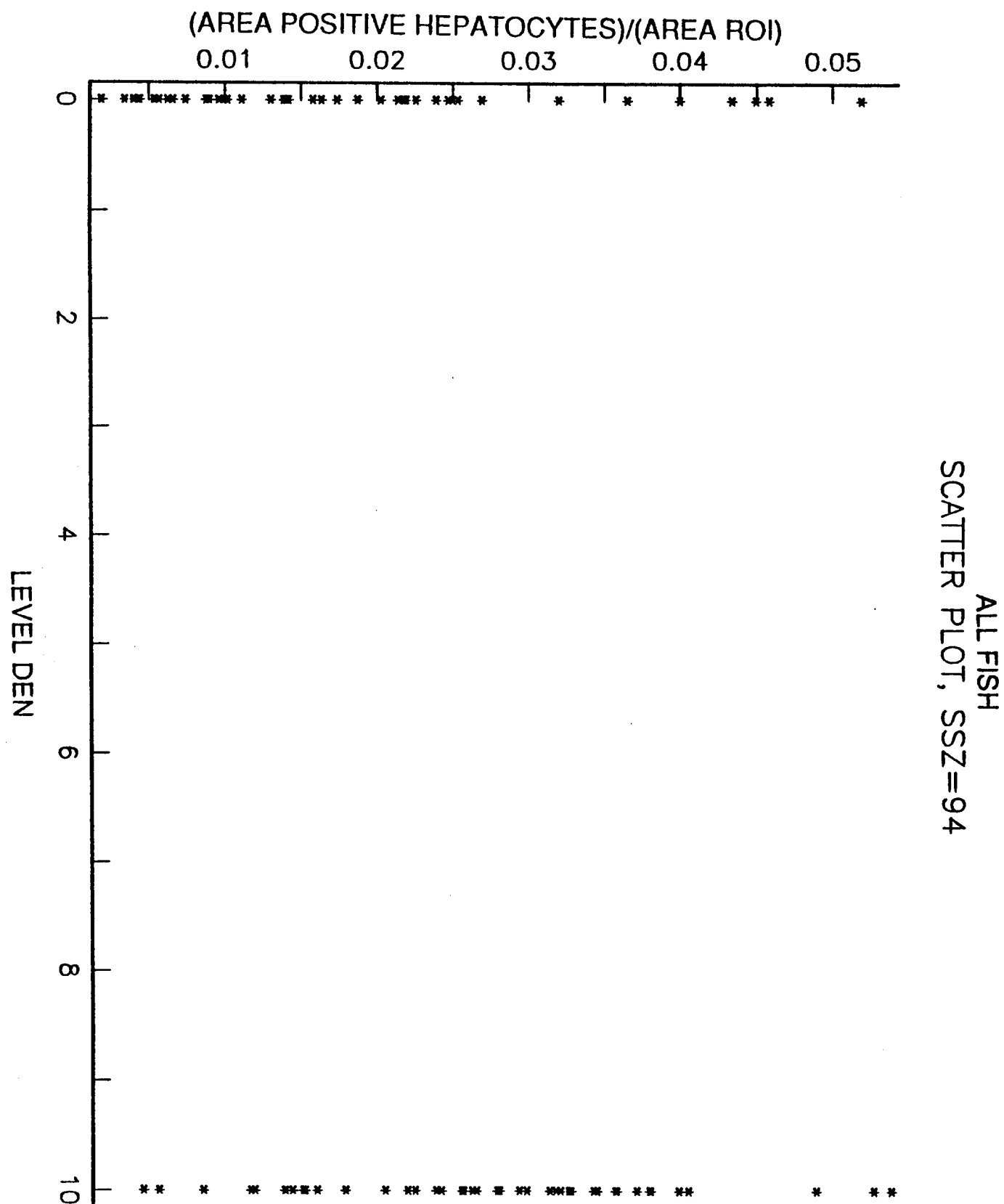


Figure 12

SCATTER PLOTS FOR SUBSAMPLES, SSZ=47

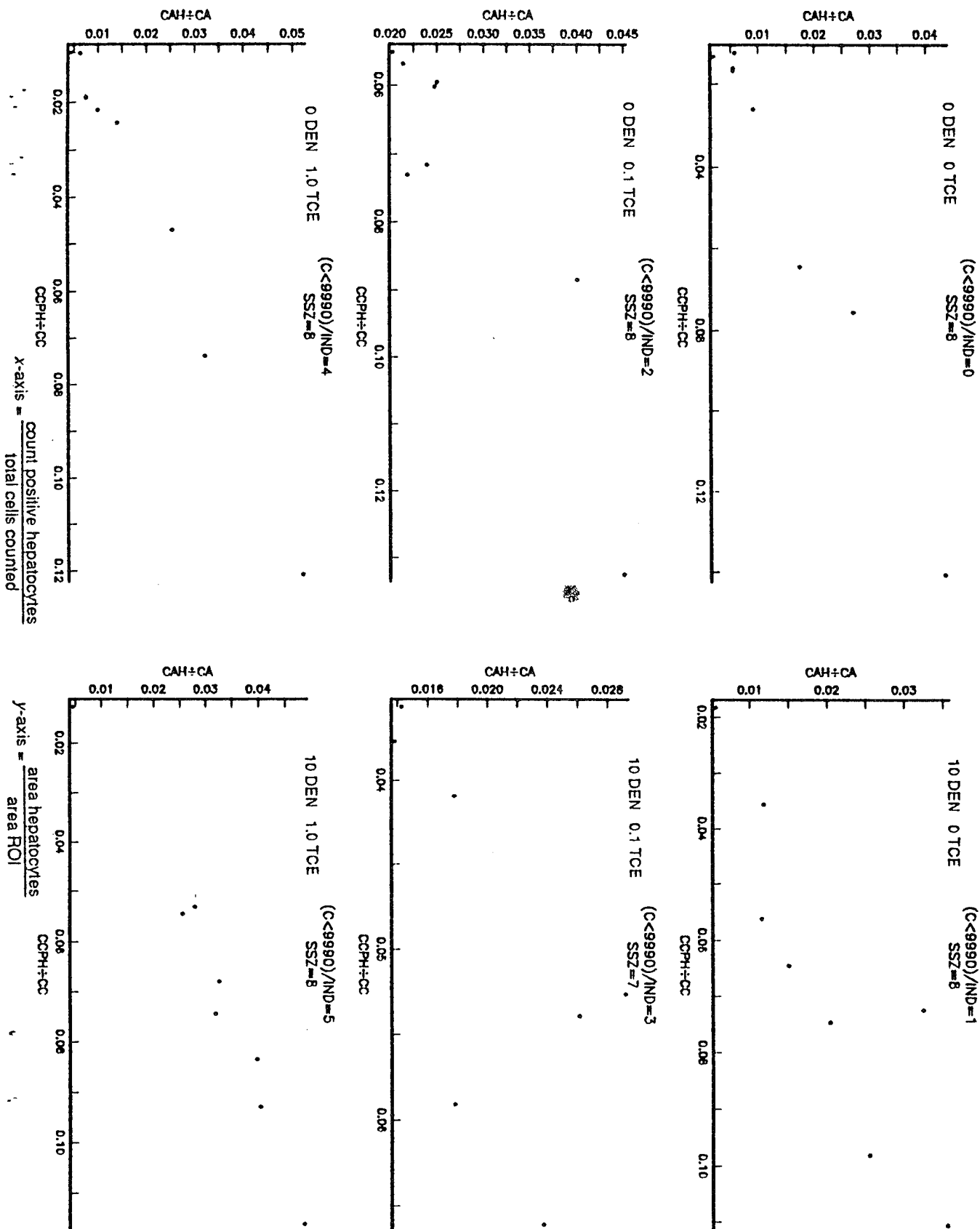


Figure 15

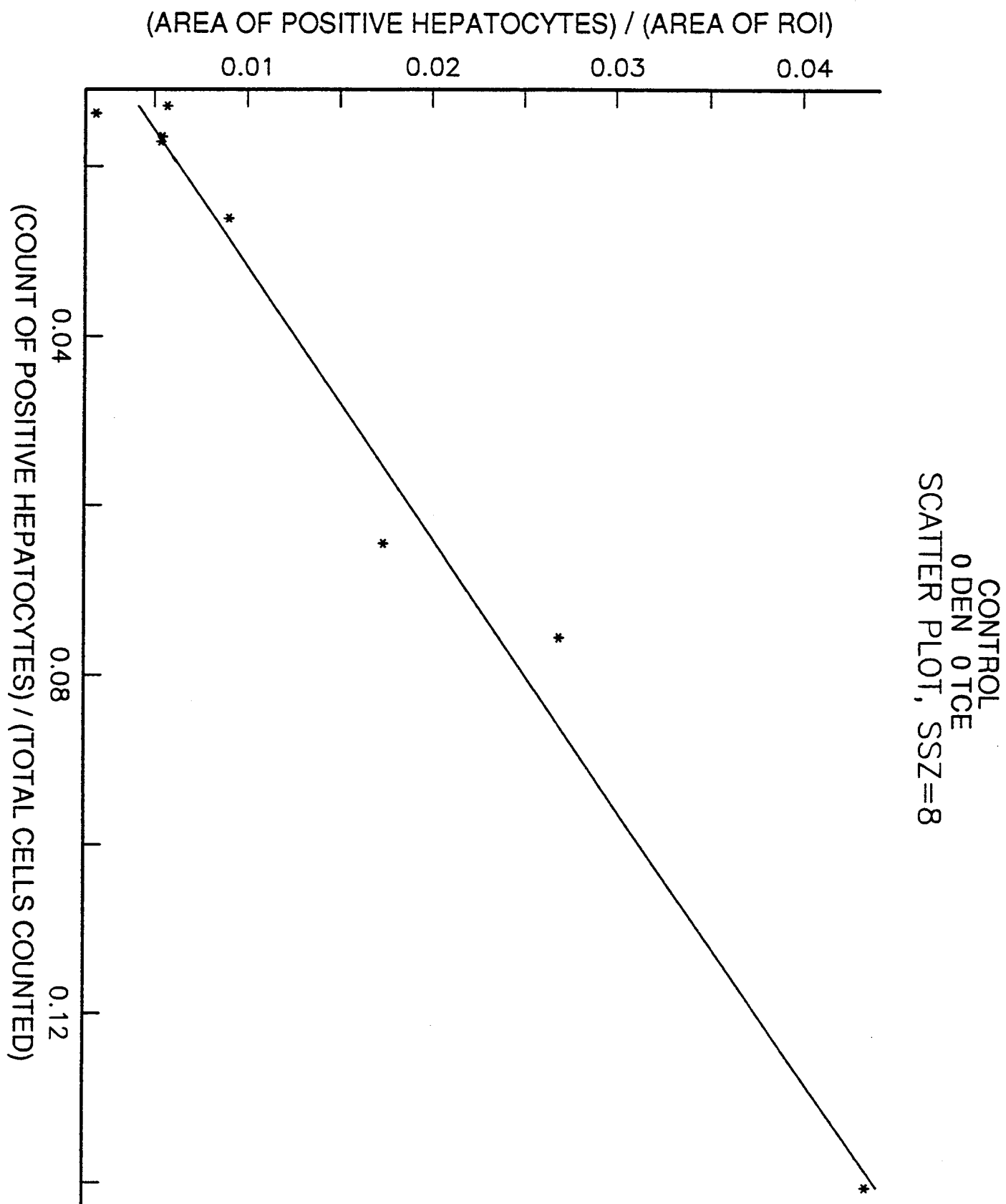


Figure 16

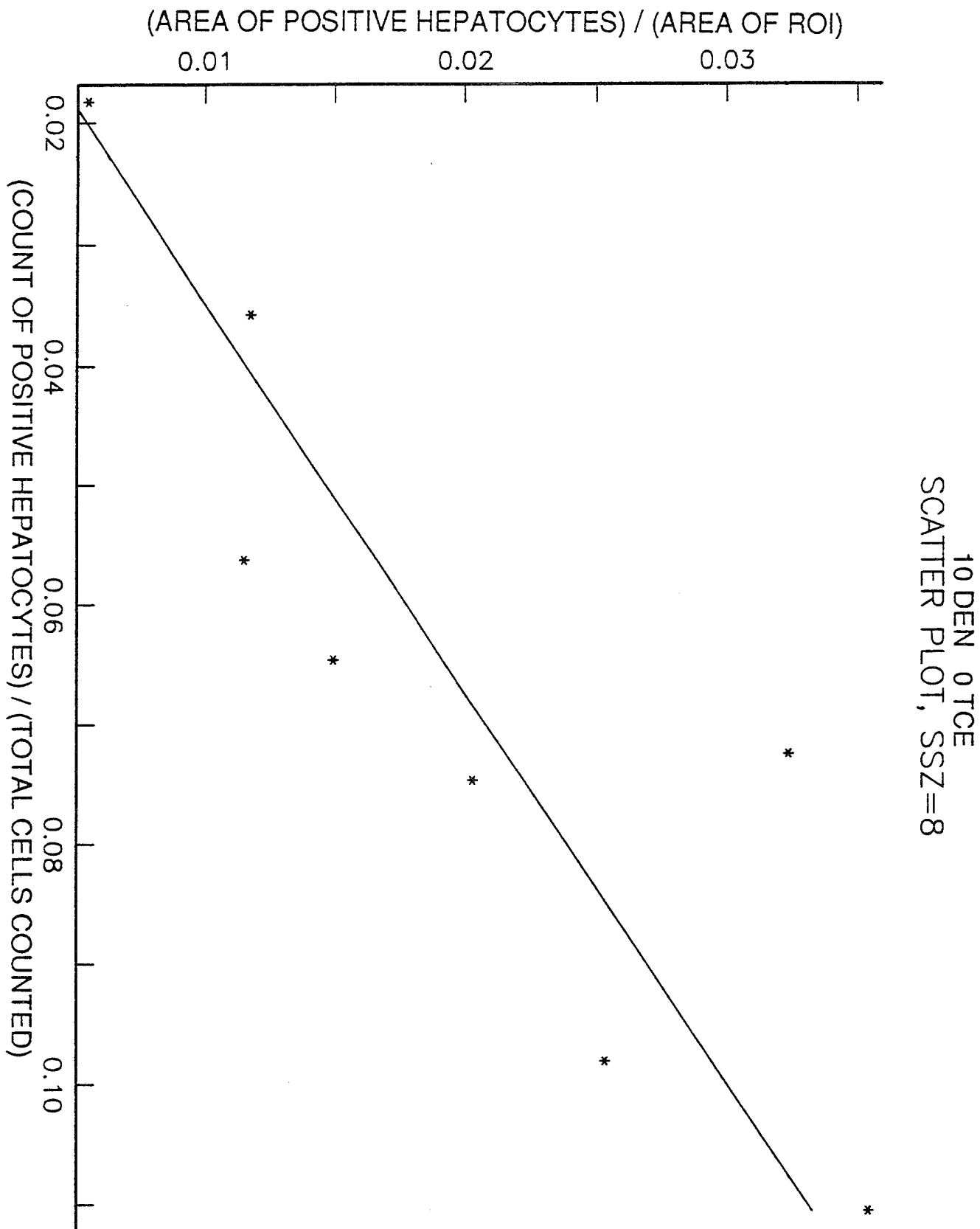


Figure 17

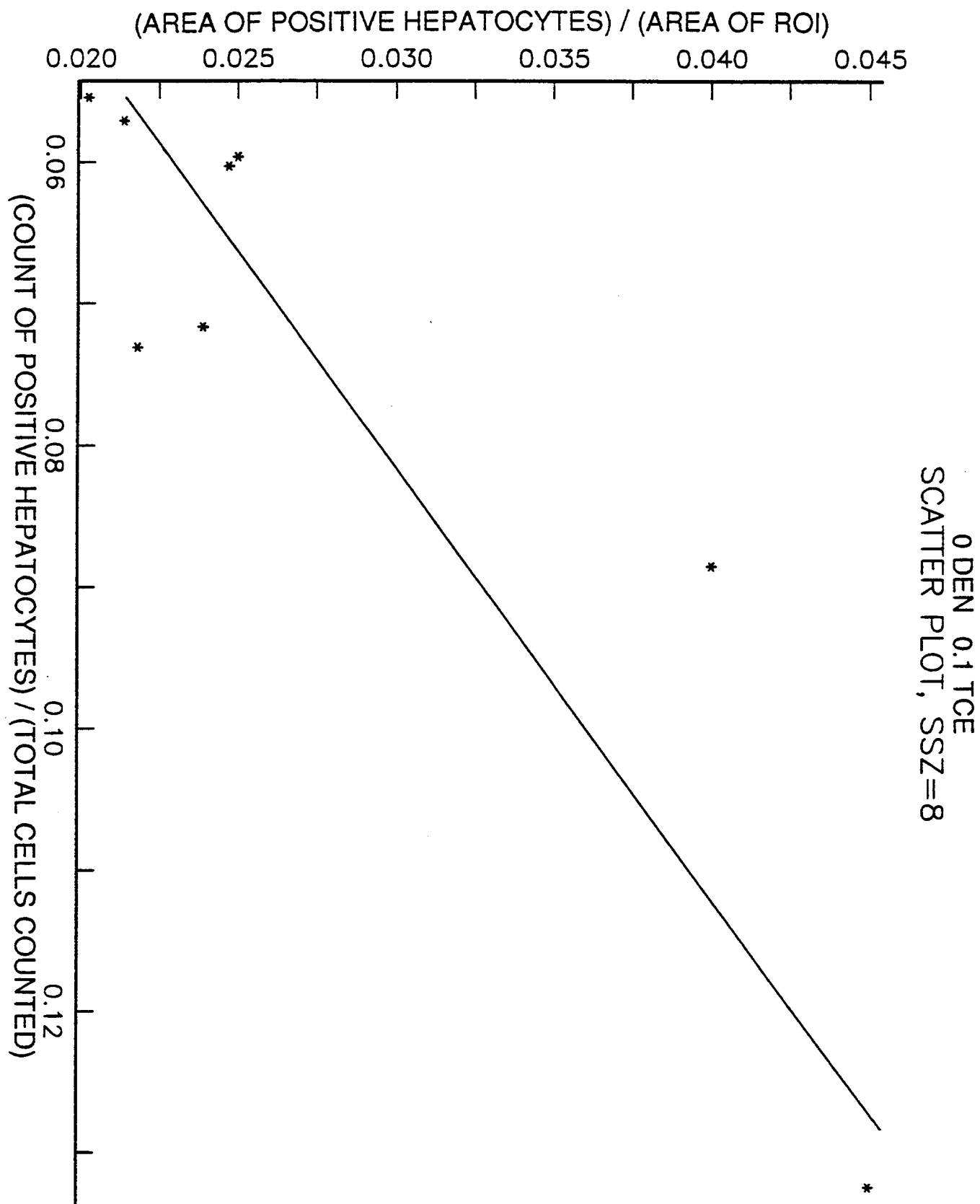


Figure 18

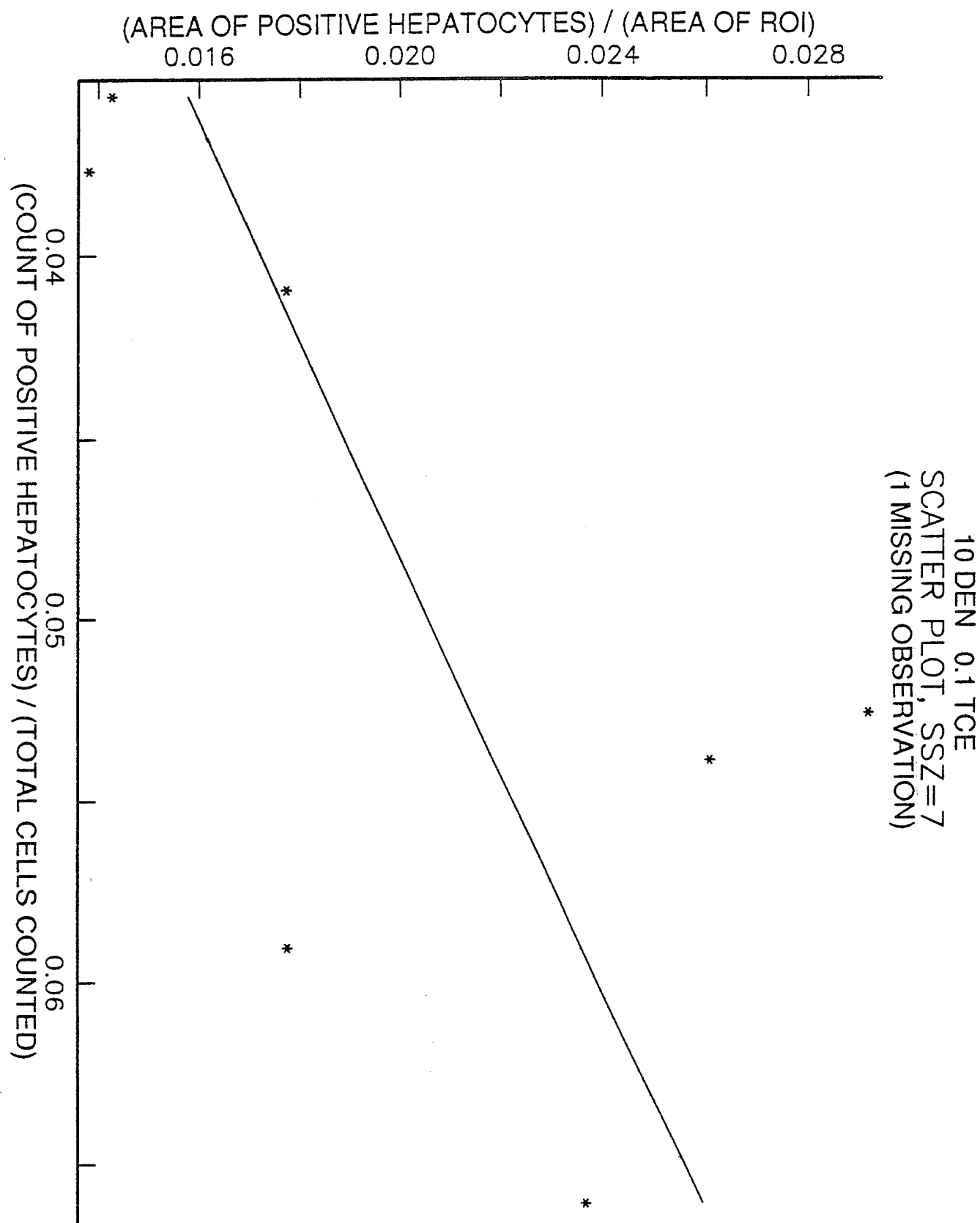


Figure 19

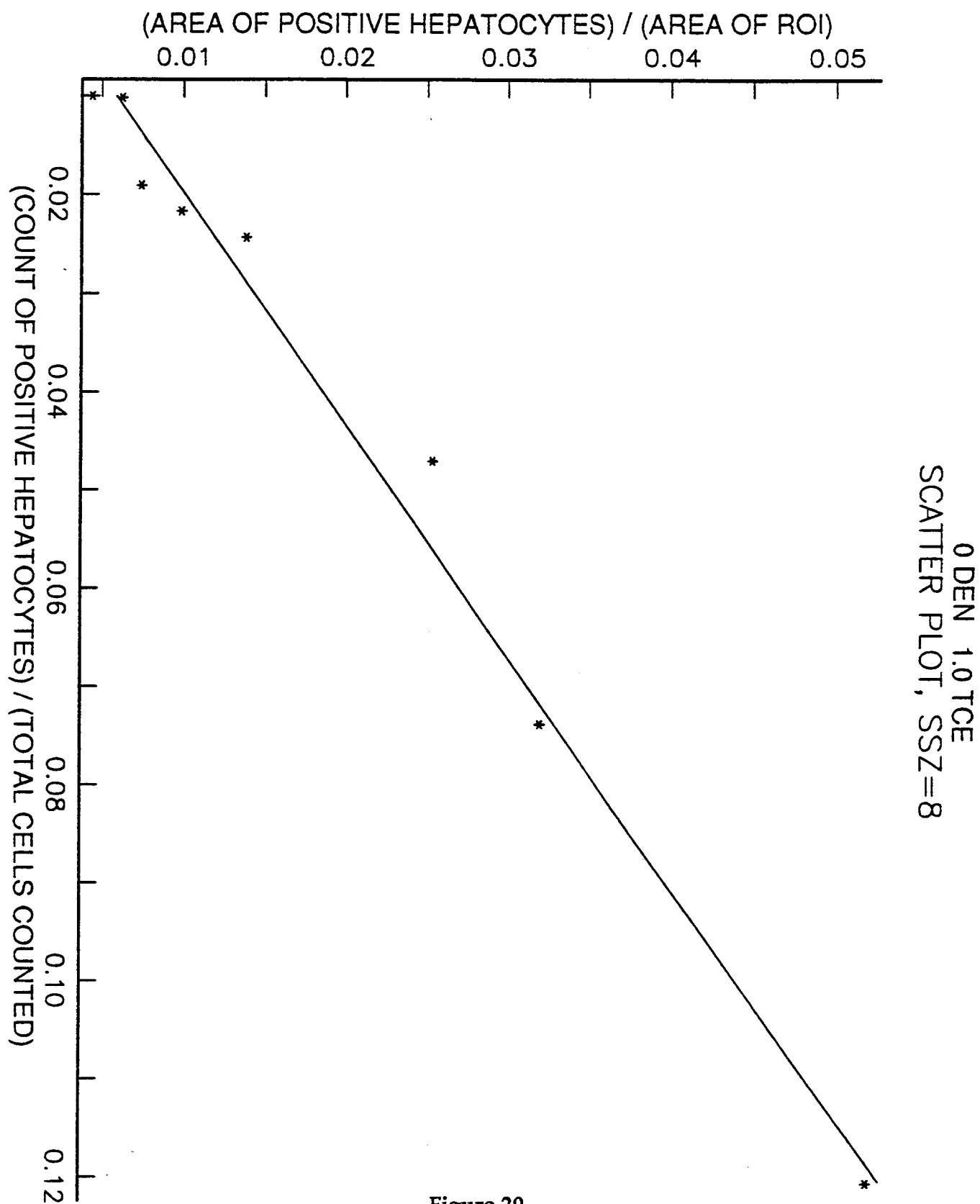


Figure 20

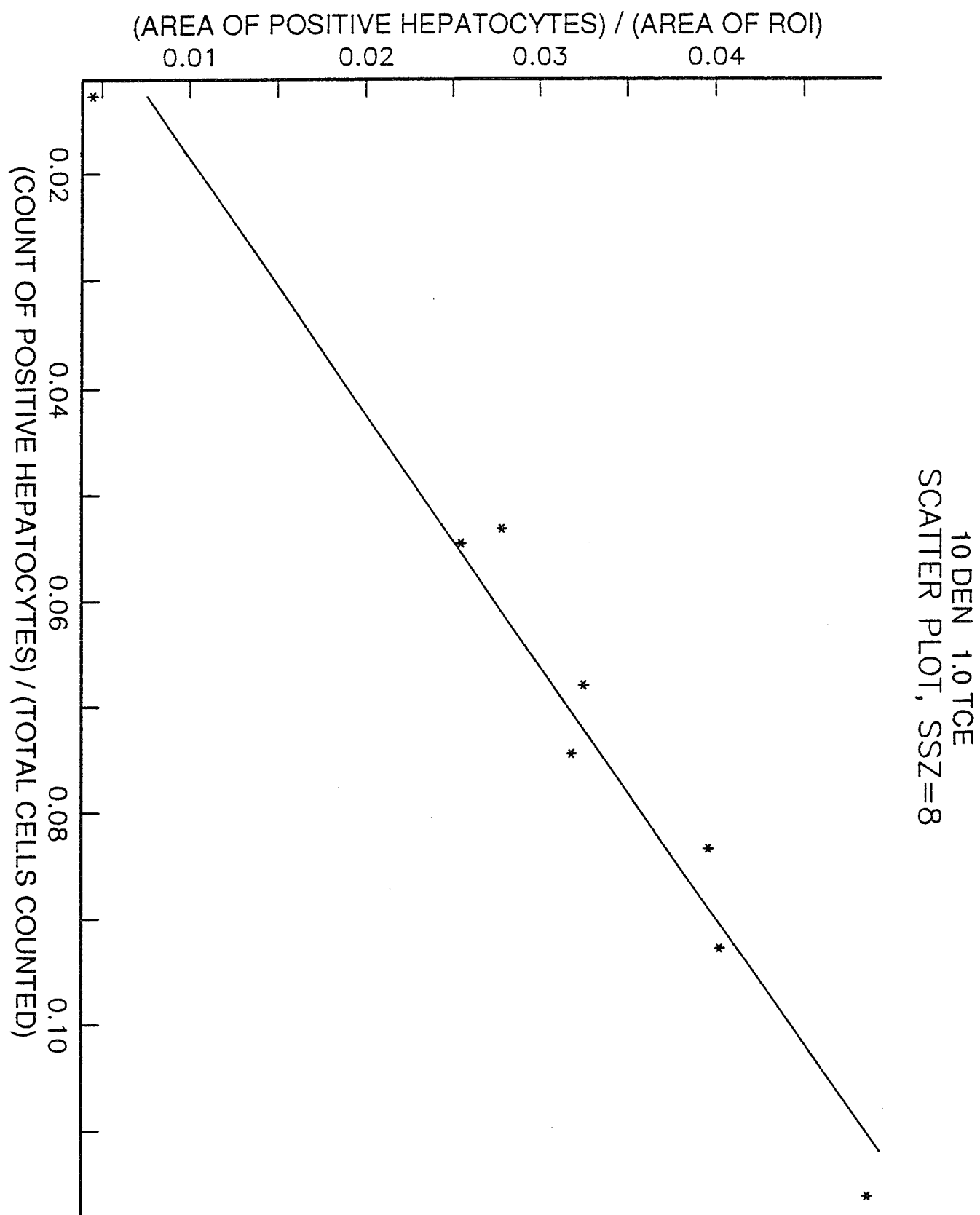


Figure 21

BOX/PERCENTILE PLOTS, SSZ=47

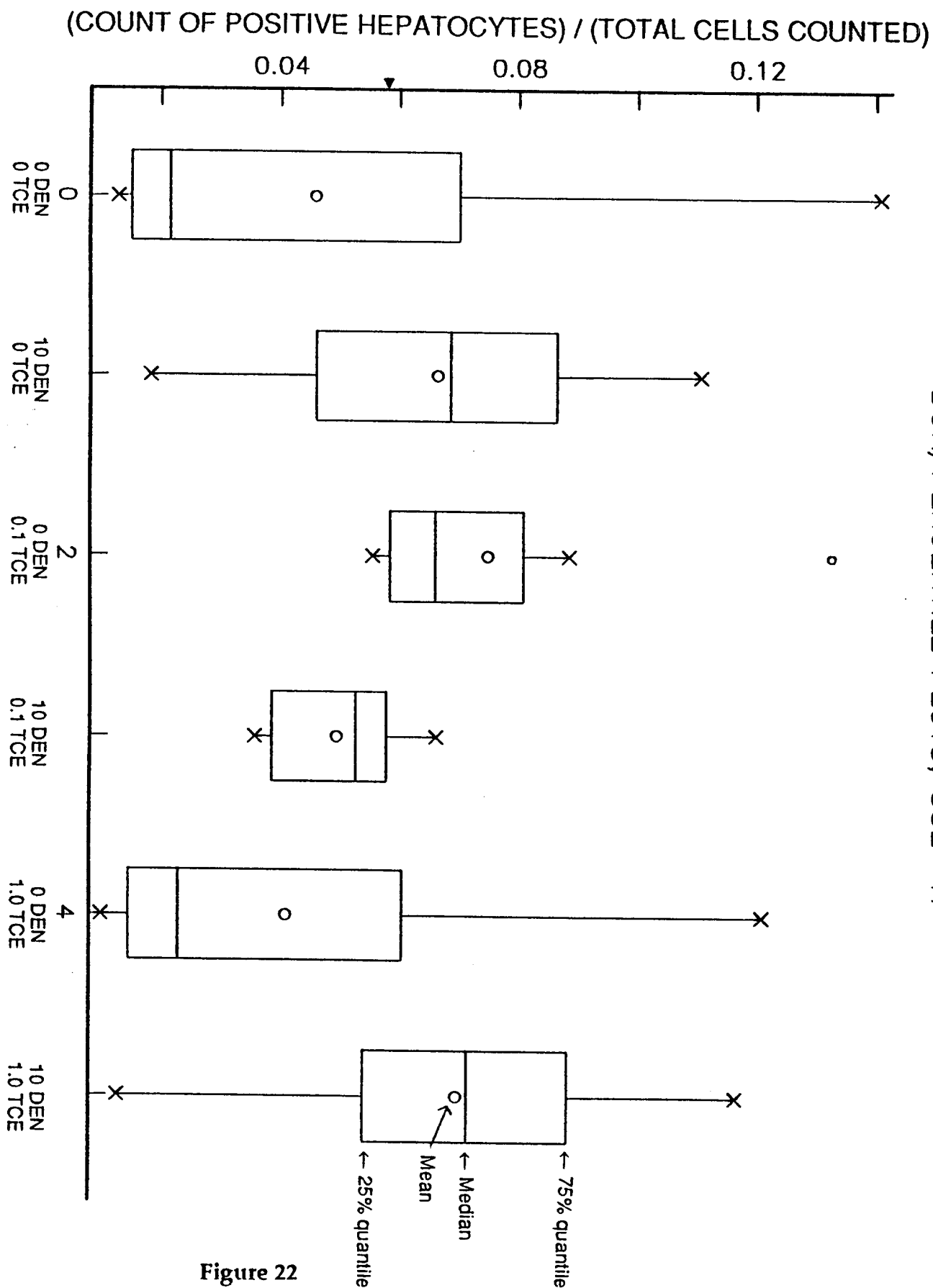


Figure 22

BOX/PERCENTILE PLOTS, SSZ=47

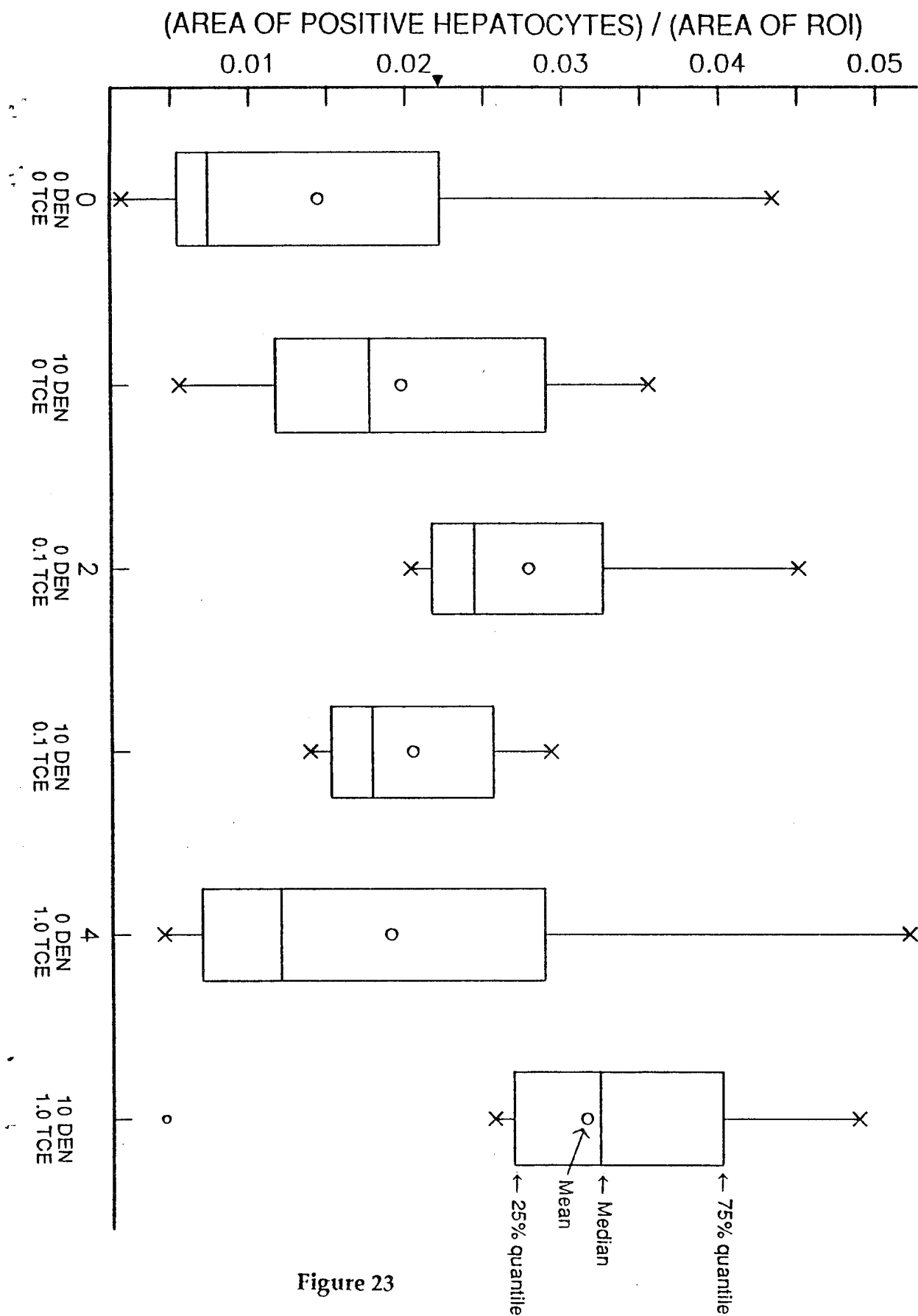


Figure 23

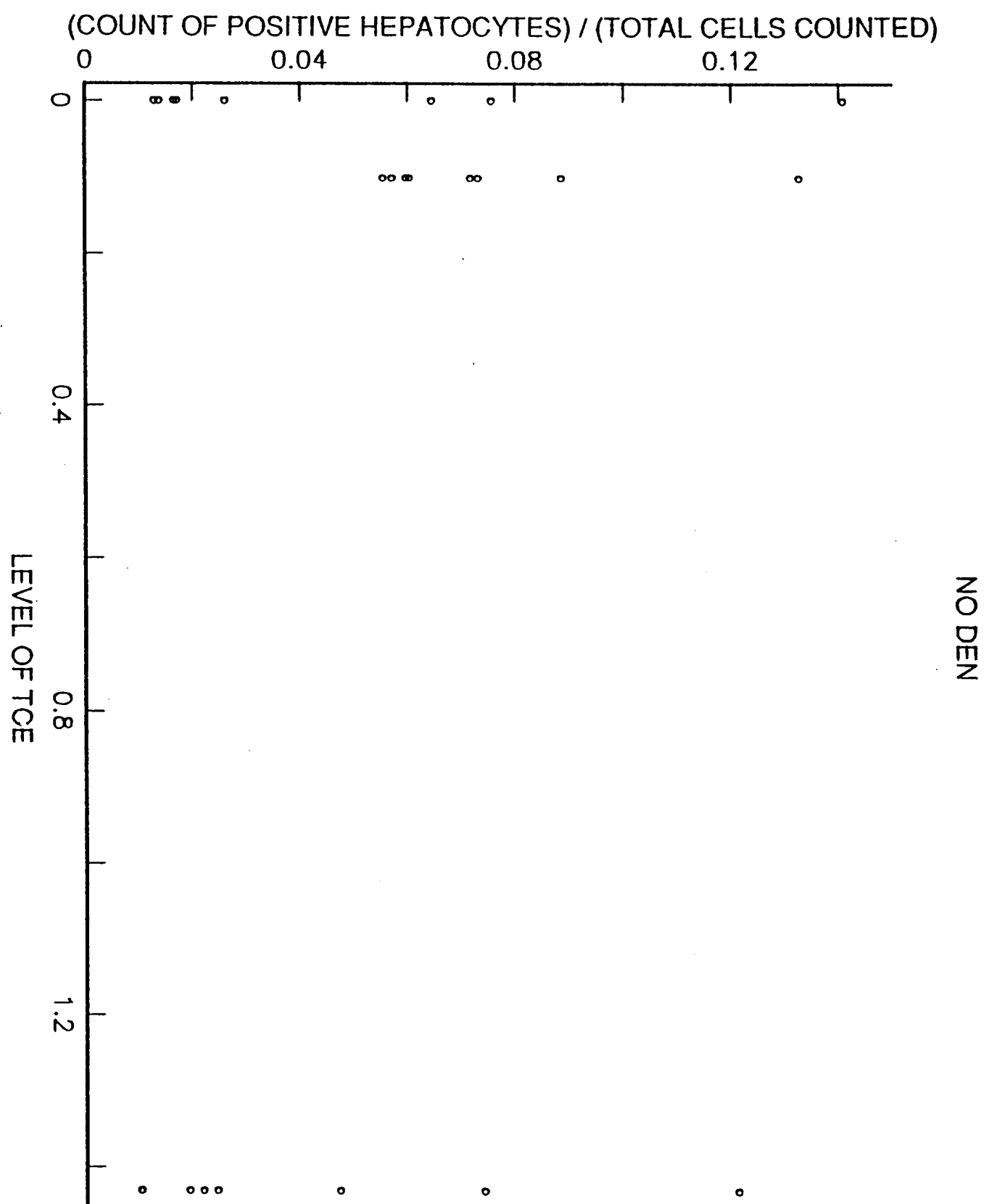


Figure 24

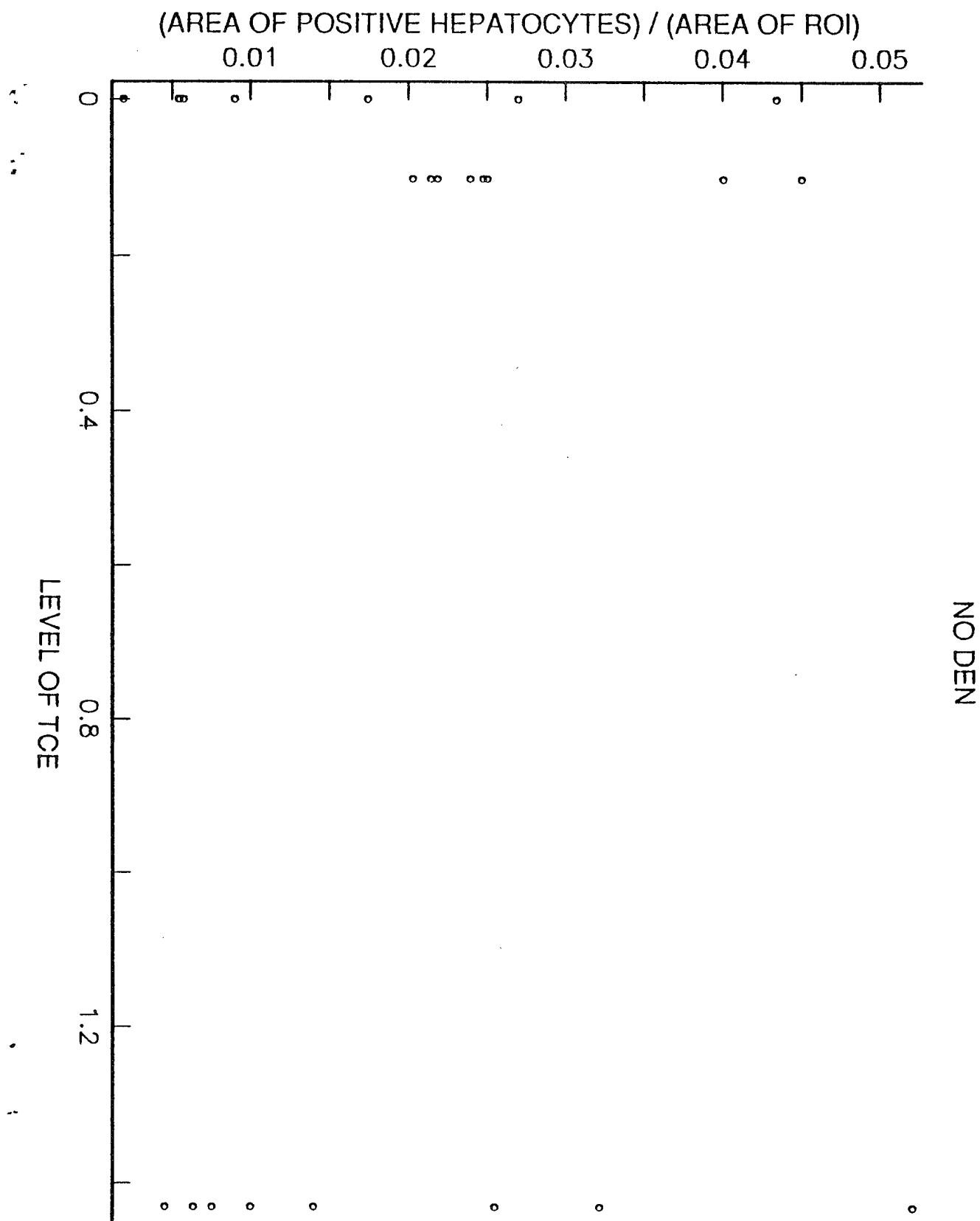


Figure 25

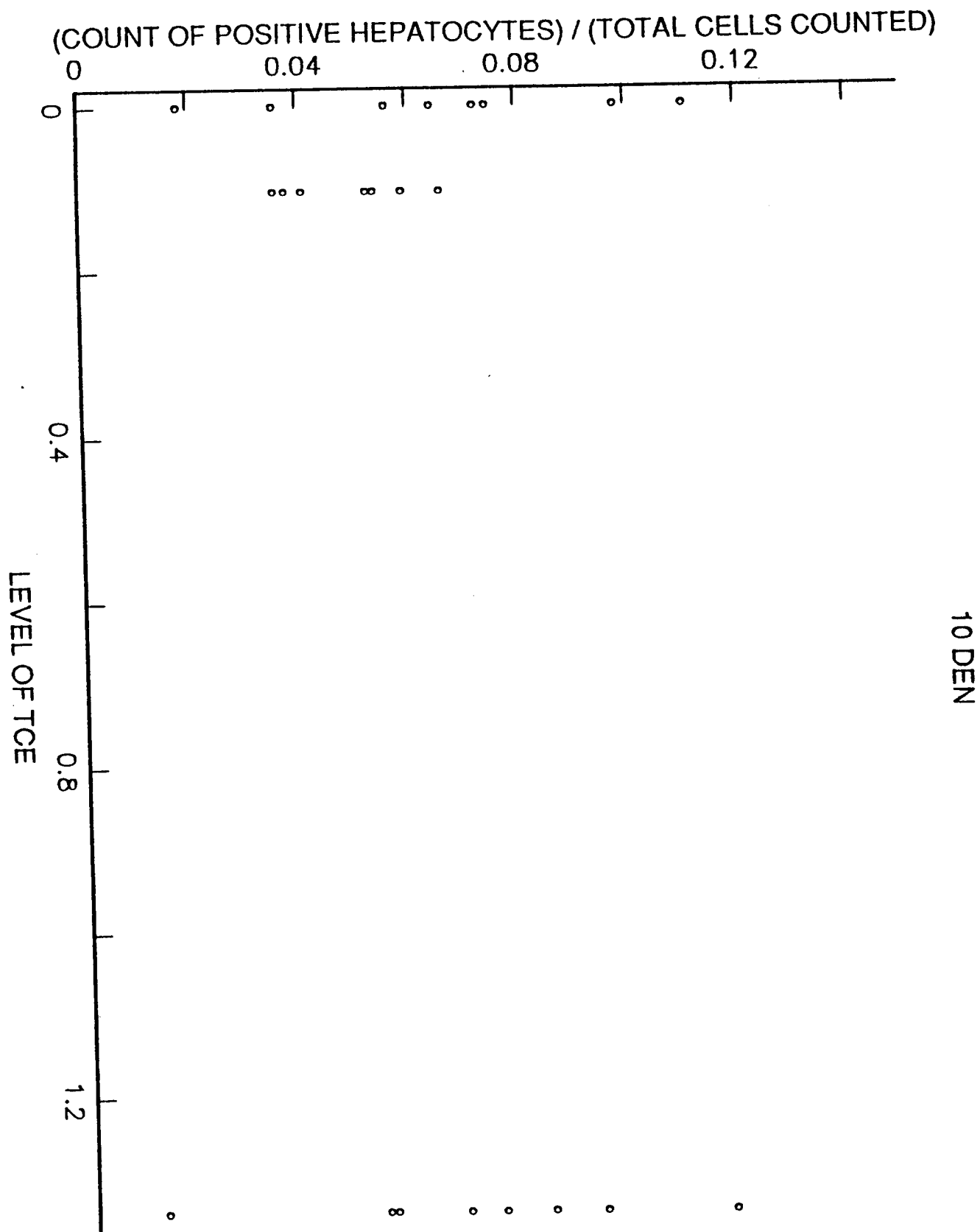


Figure 26

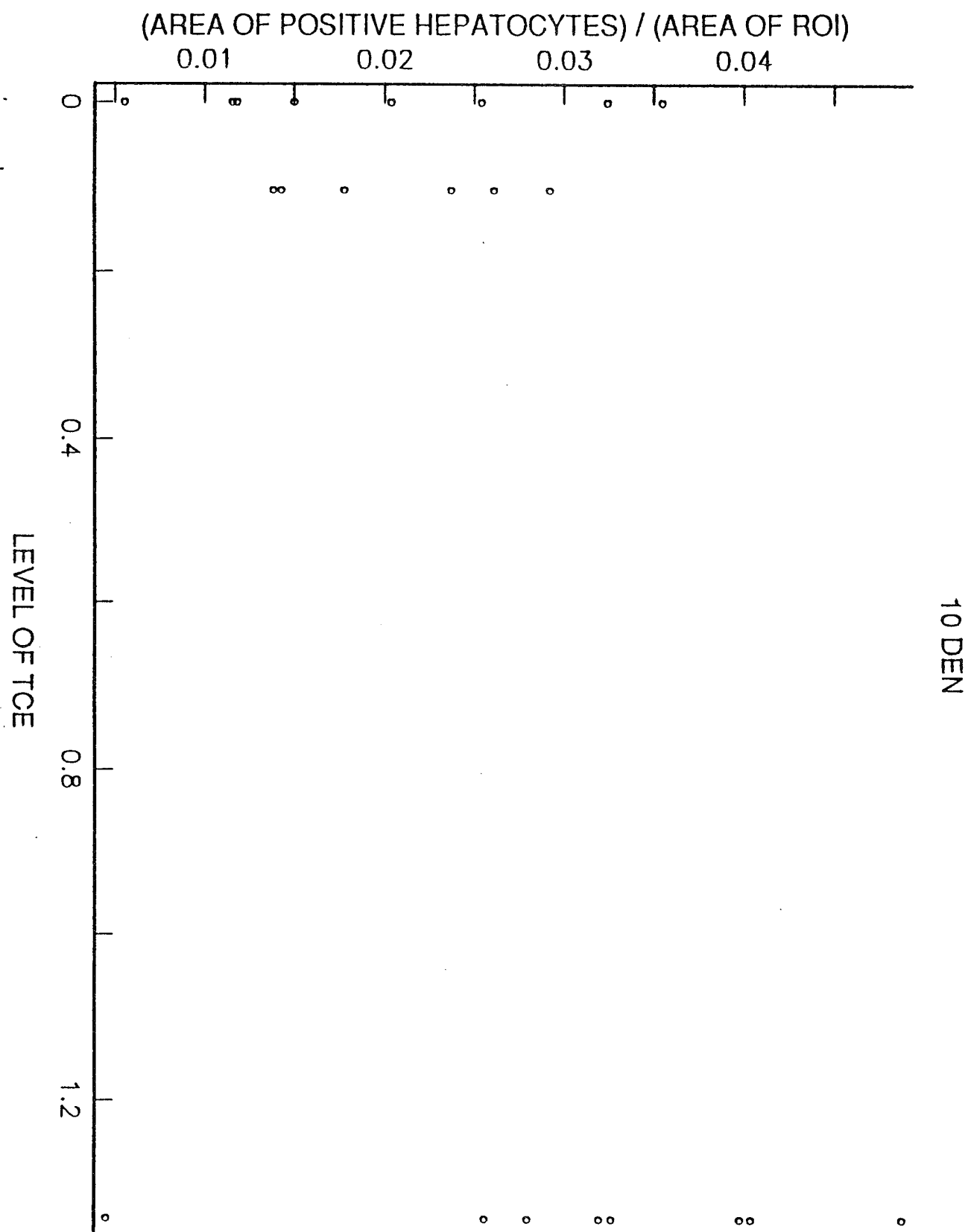


Figure 27

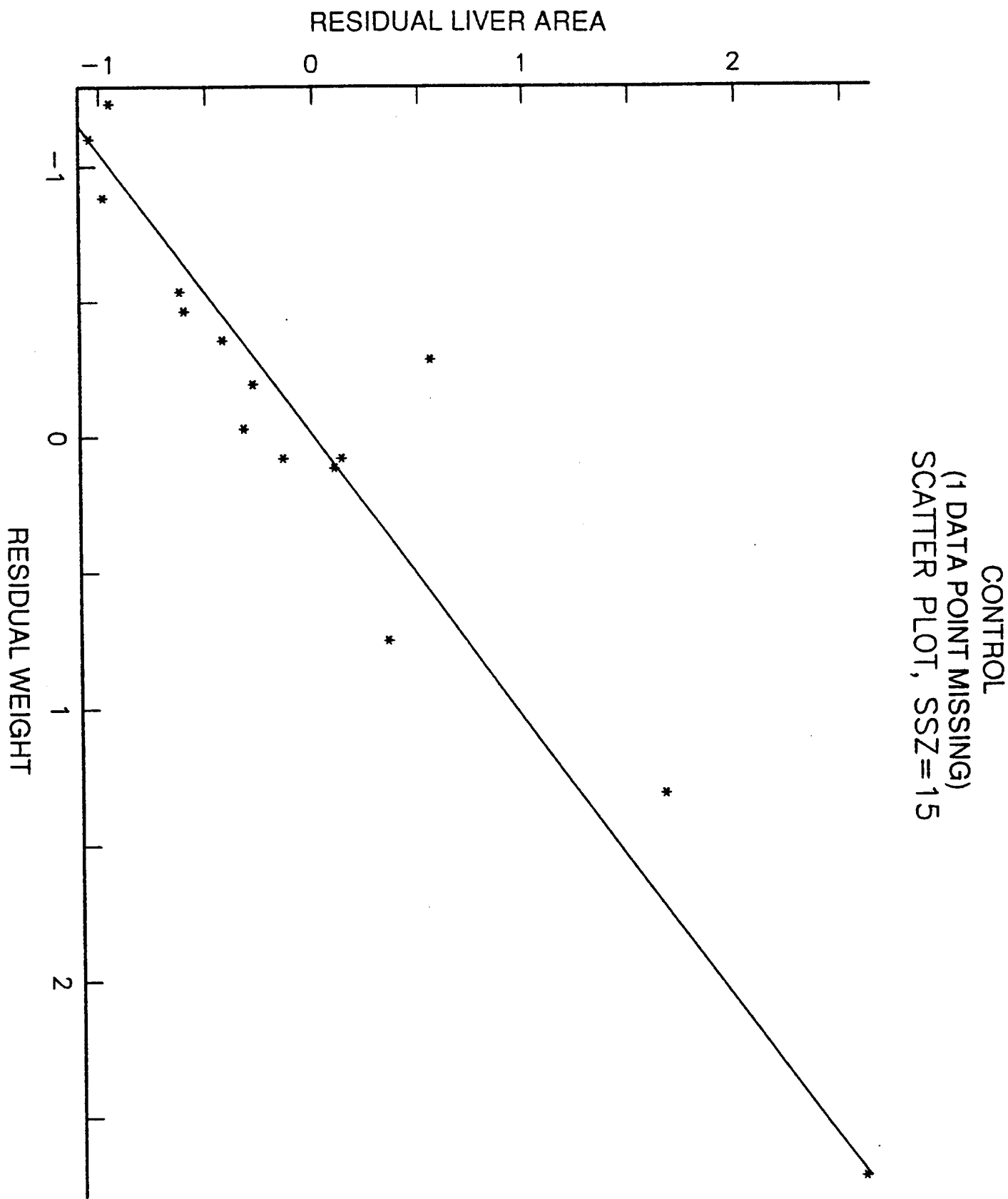


Figure 28

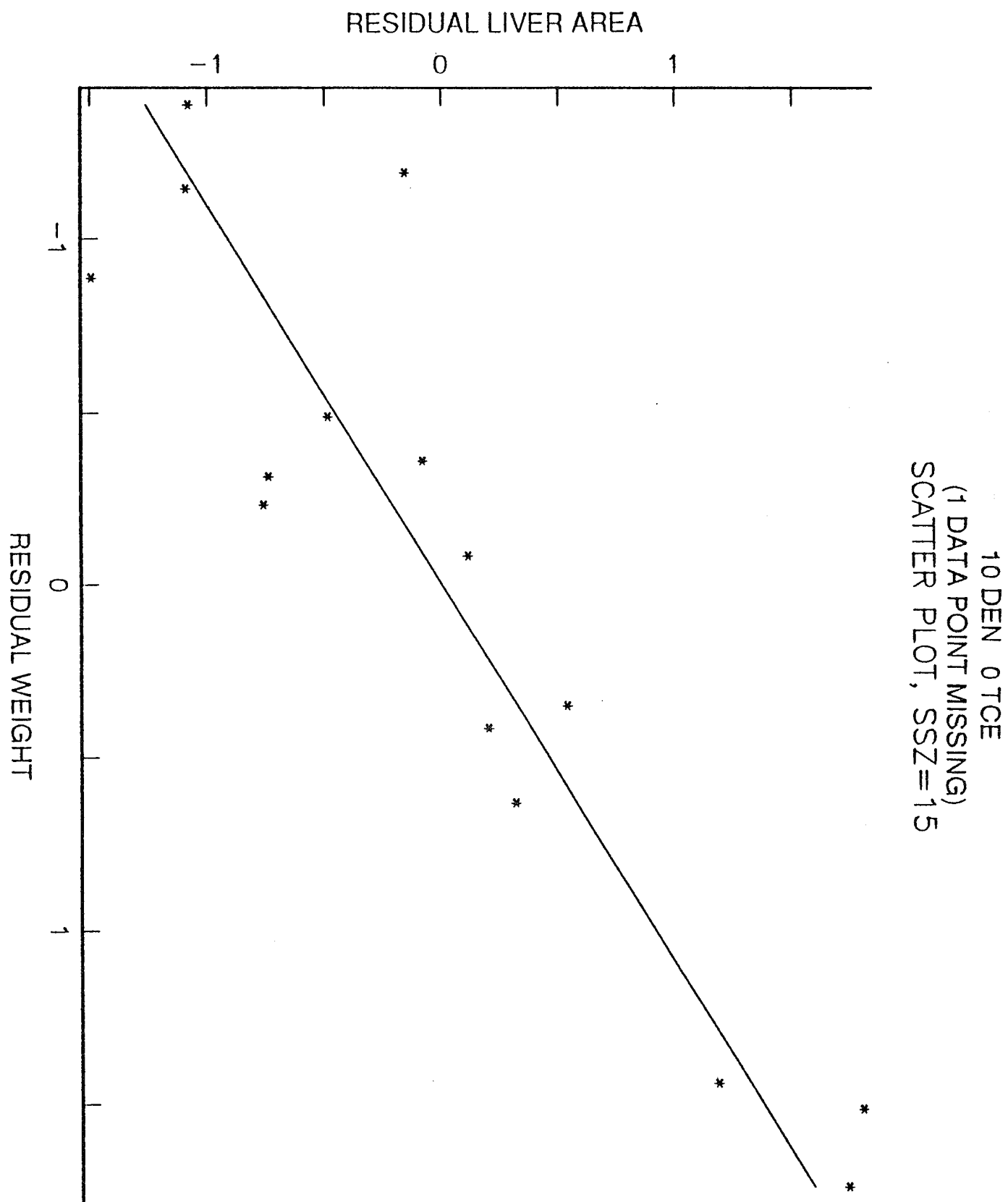


Figure 29

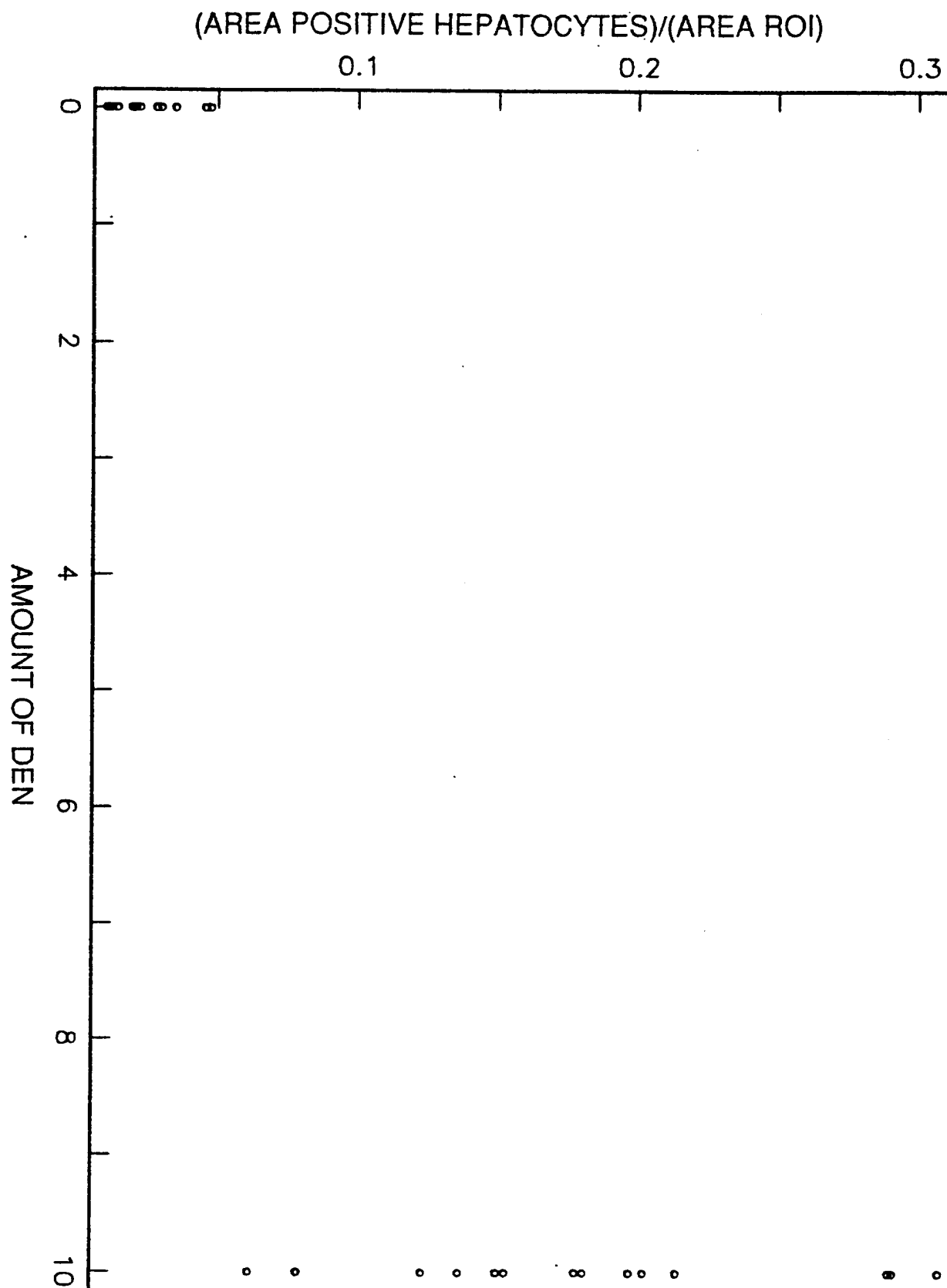


Figure 30

APPENDIX 3

COMPARISON OF AREA INDICES IN MEDAKA LIVERS FOR SACRIFICES AT DIFFERENT CONCENTRATION AND TIME COMBINATIONS USING DATA AVAILABLE 5/19/94

D. P. Gaver

P. A. Jacobs

EXECUTIVE SUMMARY

1. Mean area indices were compared for early (B) and late (D) sacrifices within tanks, so as to minimize (subtract/cancel) additive tank effects!
2. Comparison between treatment types: DEN-TCE-Controls, inevitably include between-tank effects: these have not been made as yet; this is in part because required data has just become available for analysis.
3. *Conclusion:*

The comparison of area indices in tanks 7 and 8 (0.1 TCE) and 13 and 14 (10 DEN, 1 TCE) show *depression* of area index for longer exposure time (D) as compared to shorter time (B). The pooled tank versions of the t-tests (rows 4 and 5 of Table 2) show definite significance (0.033) for pooled differences. Tanks 7 & 8 (0.1 TCE), and even more so (0.002) for tanks 13 and 14 *when a prominent outlier* (Fish 156) *is removed*.

This negative tendency is enhanced by comparison with the control tanks: Table 3, rows 4 and 5 (with outlier removed).

4. *Additional Comments*

Data behavior on individual slices for fish suggests that summarization by a robust procedure (a mid-mean or broadened median vs. a mean) would be effective and appropriate. Also, a considerable variability between fish experiencing the same treatment (tanks) is apparent, and should be adjusted for by use of a more sophisticated, e.g. hierarchical, model. Within-fish variability (across different ROIs) may well be expected, and be an important measure of response.

COMPARISON OF AREA INDICES IN MEDAKA LIVERS FOR
SACRIFICES AT DIFFERENT CONCENTRATION AND TIME
COMBINATIONS USING DATA AVAILABLE 5/19/94

D. P. Gaver

P. A. Jacobs

EXECUTIVE SUMMARY ADDENDUM

More data availability has made possible the construction of the enclosed graphs.

(a) The hollow center dots (◊) signify mean area indices; the vertically upper (*) and lower (+) marks per concentrations are upper and lower 95% confidence limits, using pooled difference data tank for tanks; this latter step tends to remove an additive tank effect.

(b) *Conclusions:*

(b-1) Rightmost two columns (0 DEN, 1 TCE vs. 10 DEN, 1 TCE): there is a not-statistically significant (at 5% level), but weakly evident, *decrease* in AI with DEN increase.

(b-2) Third and fourth-from-right columns (0 DEN, 0.1 TCE vs. 10 DEN, 0.1 TCE): there is a (weak) *increase* in mean AI with DEN increase.

(b-3) Fifth and sixth-from-right columns (100 DEN vs. 10 DEN; 0 TCE): there is a weak decrease in mean AI with DEN increase. The $\sqrt{\text{variance}}$ (width of confidence limits) seems to increase with DEN, 0 TCE.

(b-4) The control difference (effect of added time, B to D, seems to increase significantly: 0 is below lower confidence limit).

(b-5) The analyses with and without the outlier fish 156 agree reasonably well.

1. The Experimental Design

Japanese Medaka are exposed to differing levels of DEN and TCE. Each treatment has two tanks. Eight animals in each tank are sacrificed 4 Aug 1993; this is sacrifice B. Eight additional animals in each tank are sacrificed on 20 Aug 1993; this is sacrifice D.

Each sacrificed fish is exposed to BrdU for 72 hours prior to sacrifice; any cell that is in S-phase during this time will have a BrdU marker. Each sacrificed fish is frozen and sliced longitudinally. A third of the slices are stained with a microclonal antibody. This antibody stains nuclei that have been in S-phase black: these nuclei are called positive.

A region of interest (ROI) is marked on the liver of a slice; the ROI is chosen to maximize the number of hepatocytes and minimize the number of nonhepatocytes. The area of cells in the ROI is measured. The area of the positive nuclei is measured. Most fish have 5 slices; however, some apparently only have 3 slices.

2. The Data

As of 5/19/94, we have the following summary data for each animal: the area of the ROI's (respectively, positive nuclei) added over the slices, *riareasm* (respectively *riheparea*); the mean area of the ROI's (respectively, positive nuclei) over the slices, *xriarea* (respectively *xheparea*), and the standard deviation of the area of the ROI's (respectively, positive nuclei) over the slices, *sdriarea* (respectively *sdheparea*). The standard deviations of the area of positive nuclei are missing for 1/2 the fish from tanks 3, 4, 5, and 6 from sacrifice B; all of these animals apparently had 3 slices stained; in tanks 3, 4, 5, and 6 of the areas of positive nuclei are actually areas of only positive hepatocytes.

An area index is computed for each animal. For animal i

$$\tilde{a}_i = \frac{x_{heparea}}{x_{riarea}} \times 100.$$

There are additional missing observations. Later we obtained the raw slice data for the experiment. Most fish for the later data seemed to have 5 slices considered. Thus, there may be some recording errors in the $x_{heparea}$ and x_{riarea} in the data available at 5/19/94.

3. The Question

Is there a difference in area indices between sacrifice B and D?

In more detail we state this as follows. We are in possession of sampled values of area indices from fish that have undergone various treatment combinations, these being (at least): x DEN, y TCE, z Exposure time, t Tank. Assume that those sample values can be combined for a given treatment combination to provide a meaningful estimate of a true area index associated with the treatment. Is there evidence from the estimates for consistent difference between the true area indices associated with treatments?

As of 5/19/94, we have data from sacrifices B and D for 7 treatment groups: the control, 10 mg/ℓ DEN, 100 mg/ℓ DEN, (0 mg/ℓ DEN with 0.1 mg/ℓ TCE), (10 mg/ℓ DEN with 0.1 mg/ℓ TCE), (0 mg/ℓ DEN with 1 mg/ℓ TCE), and (10 mg/ℓ DEN with 1 mg/ℓ TCE). As a result, we will restrict our attention to these groups.

4. A Graphical Data Analysis

Figure 1 displays the area indices divided by 100 by tank for both sacrifices. Figure 2 displays box plots of the same data. The area indices are larger and have greater variability for the 100 mg/ℓ DEN treatment group. There is the indication

that area indices may tend to be larger for sacrifice D in treatment groups control, 10 mg/ ℓ DEN, and 100 mg/ ℓ DEN. There is the indication that area indices are smaller for sacrifice D for the treatment groups that are exposed to TCE; 0 mg/ ℓ DEN with 0.1 mg/ ℓ TCE and 10 mg/ ℓ DEN with 1 mg/ ℓ TCE.

5. Statistical Analysis Based on Ranks

One way to compare two samples is to use the ranks of the combined samples; the resulting test is called a Mann-Whitney-Wilcoxon test or a rank sum test; Gibbons (1985), Mosteller and Rourke (1973). To compute the statistics, the two samples are combined and ordered from smallest to largest and ranked; the smallest observation is ranked 1. The sum of the ranks for each sample is computed. The null hypothesis for the test is that the two samples come from the same distribution.

The area indices from sacrifices B and D for each tank are combined. The combined indices for each tank are ordered from smallest to largest and ranked. The sums of the ranks for data from sacrifice B (respectively D) are computed. The results appear in Table 1.

The null hypothesis for the rank sum test is that the area indices from sacrifices B and D come from the same distribution. Since one half animals in tanks 3, 4, 5, 6 for sacrifice B apparently have 3 slices examined while the other animals have 5 slices examined, the variability of the area indices for the animals may be different.

The p -values of the sum of ranks are less than 0.05 for tanks 1, 8, 13, and 14. Since tanks 13 and 14 belong to the treatment group of 10 mg/ ℓ DEN and 1 mg/ ℓ TCE, there is a strong indication that the treatment results in a lower index for sacrifice D than for sacrifice B. One tank (tank 1) for the control has a significant

p -value, the other does not. One tank (tank 8) in the treatment group 0 DEN, 0.1 mg/ℓ TCE has a significant p -value, the other does not.

6. Statistical Analysis Based on Moments

Moments, especially means, are often a useful summary of data. In this section we describe an analysis of the data using moments.

The sample mean of the area indices $\bar{a}_{B,j}$ (respectively $\bar{a}_{D,j}$) and sample variance of the area indices $s_{B,j}^2$ (respectively $s_{D,j}^2$) for sacrifice B (respectively sacrifice D) for tank j are computed.

The difference of the means

$$d_j = \bar{a}_{D,j} - \bar{a}_{B,j}$$

and an estimate of the variance of the difference

$$v(d)_j^2 = \frac{s_{B,j}^2}{n_{B,j}} + \frac{s_{D,j}^2}{n_{D,j}}$$

is computed where $n_{B,j}$ (respectively $n_{D,j}$) is the number of animals for sacrifice B (respectively sacrifice D) for tank j . This variance estimate does not assume that the tank area index variances are the same for sacrifices B and D.

The difference of the mean area index between sacrifices B and D over the two tanks in each treatment group and an estimate of its variance is computed; for example, tanks 1 and 2 are used to obtain a mean (D-to-B) difference for the control group μ_c and an estimated variance in the following manner.

$$\mu_c = \frac{1}{2}[d_1 + d_2]$$

$$v_c(d)^2 = \frac{1}{4}[v(d)_1^2 + v(d)_2^2]$$

If the assumption is made that area indices for each tank have the same population variance within a treatment group, then an estimate of the variance of the area index is

$$s^2(A) = \frac{1}{(n_{B,1} + n_{B,2} + n_{D,1} + n_{D,2} - 4)} \left(\sum_{j=1}^2 \sum_i (a_{B,j}(i) - \bar{a}_{B,j})^2 + \sum_{j=1}^2 \sum_i (a_{D,j}(i) - \bar{a}_{D,j})^2 \right)$$

where $a_{B,j}(i)$ is the i^{th} area index for sacrifice B from tank $j = 1, 2$ (first and second) for a treatment group. An estimate of the variance of d_j for this treatment group would be

$$v(c)_j^2 = \left(\frac{1}{n_{B,j}} + \frac{1}{n_{D,j}} \right) s^2(A).$$

An estimate of the variance of the mean of the mean differences between sacrifice B and D for the two tanks of the control

$$\mu_c = \frac{1}{2} [d_1 + d_2]$$

is

$$v_c(c)^2 = \frac{1}{4} [v(c)_1^2 + v(c)_2^2].$$

Under the null hypothesis of no difference between the sacrifices, the statistic $\mu_c / \sqrt{v_c(c)^2}$ has an approximate t -distribution with approximate-conservative degrees of freedom $(4 \min(n_{B,1}, n_{B,2}, n_{D,1}, n_{D,2}) - 4)$.

Under the same null hypothesis we assume $\mu_c / \sqrt{v_c(d)^2}$ has the same approximate t -distribution.

Figure 3 displays the mean differences plus/minus 2 standard deviations for the standard deviations computed not using the assumption of equal variances.

Figure 4 displays the mean differences plus/minus 2 standard deviations computed using the assumption of equal variance across tanks.

Figures 3 and 4 appear about the same. The statistics for both computations appear in Table 2 with the 2-sided p -values. The mean area indices for the control, 10 mg/ ℓ DEN, and 0 mg/ ℓ DEN with 0.1 mg/ ℓ TCE are significant.

The data displayed in Figure 1 indicates that the area index of fish 156 in the treatment group 10 mg/ ℓ DEN with 1.0 mg/ ℓ TCE is unusually large. Figures 5 and 6 display the difference of mean area indices of sacrifice B and D by treatment without fish 156. The t -statistics without Fish 156 are displayed in parenthesis in Table 2. Note that without Fish 156 there is a statistically significant difference between the mean area indices for sacrifice D and B for the treatment group 10 mg/ ℓ DEN with 1.0 mg/ ℓ TCE. Hence the mean area index for sacrifice D is less than that for sacrifice B for this treatment group.

Table 4 displays statistics for the difference of mean area indices between sacrifices B and D for each tank with 2-sided p -values. This Table should be compared to Table 1. Comparing the p -values of both Tables indicates that while there is general agreement whether or not there is a significant difference, it is not complete.

Table 3 displays statistics for the difference of mean area indices for sacrifices B and D for each non-control treatment minus the difference of mean area indices for sacrifices B and D for the control; the results without Fish 156 are displayed in parentheses. The 2-sided p -values suggest 1) the 0.1 mg/ ℓ TCE with no DEN results in the difference of the mean area index for B and D being more negative than that for the control; 2) the treatment of 10 mg/ ℓ DEN with 1 mg/ ℓ TCE results in the mean area index being more negative than that of the control. If Fish 156 is removed the treatment group of 10 DEN with 1 mg/ ℓ TCE is even more significantly different from the control.

The t -statistic for difference of mean area indices between sacrifices B and D for treatment group 10 mg/ ℓ DEN with 0.1 mg/ ℓ TCE minus the mean difference for the treatment group 0 mg/ ℓ DEN with 0.1 mg/ ℓ TCE is 1.89 (without the assumption of unequal variances) or 2.19 (if equal variances are assumed) with approximate degrees of freedom 48; the two-sided p -values are $P\{|t_{48}| > 1.89\} = 0.06$ and $P\{|t_{48}| > 2.19\} = 0.03$.

Thus, there is an indication of a significant difference between the difference in mean area indices between sacrifices B and D for treatment with 0.1 mg/ ℓ DEN and whether or not fish were exposed to DEN; exposure to DEN increases the mean difference.

The t -statistic for difference of mean area indices between sacrifices B and D for treatment group 10 mg/ ℓ DEN with 1 mg/ ℓ TCE minus the mean difference for the treatment group 0 mg/ ℓ DEN with 1 mg/ ℓ TCE is -1.38 (without the assumption of unequal variances) or -1.67 (with the assumption of equal variances) with an approximate degrees of freedom 48. If the outlying fish 156 is removed the t -statistics become -3.17 (without equal variance assumption) and -3.53 (with equal variance assumption) with approximate degrees of freedom 40. The two-sided p -values for these statistics are

$$P\{|t_{48}| < -1.38\} = 0.17 \text{ and } P\{|t_{48}| < -1.67\} = 0.10.$$

$$P\{|t_{40}| < -3.17\} = 0.003 \text{ and } P\{|t_{40}| < -3.53\} = 0.001.$$

Hence, if fish 156 is included there is no significance difference between the change in mean area indices between sacrifices B and D for the treatment groups having 1 mg/ ℓ TCE and either no DEN or 10 mg/ ℓ DEN. If fish 156 is excluded then there is a significance difference with the decrease in mean area index for sacrifice D compared to sacrifice D being greater for the treatment group

10 mg/ℓ DEN with 1.0 mg/ℓ TCE than for the treatment group 0 DEN with 1.0 mg/ℓ TCE.

REFERENCES

- Gibbons, J. D., *Nonparametric Methods for Quantitative Analysis*, Second Edition, American Sciences Press, Columbus, OH, 1985.
- Mosteller, F. and R. E. K. Rourke, *Sturdy Statistics: Nonparametrics and Order Statistics*, Addison-Wesley, Menlo Park, CA, 1973.
- IBM Corporation. A Graphical Statistical System (AGSS).

TABLE 1
Ranks of Area Indices for Sacrifices B and D

Tank	Treatment		Number of Fish		Rank		<i>p</i>
	DEN	TCE	B	D	B	D	
1	0	0	8	8	45	91	0.007
2	0	0	8	7	56	64	0.198
3	10	0	8	8	61	75	0.253
4	10	0	8	7	56	64	0.198
5	100	0	8	8	52	84	0.052
6	100	0	8	8	59	77	0.191
7	0	0.1	8	8	71	65	0.399
8	0	0.1	7	8	71	49	0.047
9	10	0.1	7	8	59	61	>0.522
10	10	0.1	8	7	55	65	0.168
11	10	1	8	8	70	66	0.439
12	10	1	8	8	78	58	0.164
13	10	1	8	8	90	46	0.010
14	10	1	8	7	79	41	0.047

The entries labeled *p* in the table are the cumulative probability from each extreme to the value of the statistic for the X-sample for the given sample size $m \leq n$ (m is the size of the X-sample; n is the size of the Y-sample). Left-tail probabilities are given for $T_X \leq m(N + 1)/2$ and right-tail probabilities for $T_X \geq m(N + 1)/2$ where $N = m + n$; from Gibbons (1985).

TABLE 2
Difference of Mean Area Indices Between Sacrifices B and D
for Each Treatment
with Fish 156
(without Fish 156)

Treatment		Approximate Degrees of Freedom	Different Variance Mean $t = \frac{\text{Mean}}{\text{Std. Error}}$	<i>p</i> -value $P\{ T > t\}$	Common Variance Mean $t = \frac{\text{Mean}}{\text{Std. Error}}$	<i>p</i> -value $P\{ T > t\}$
DEN	TCE					
0	0	24	3.03	0.0058	3.09	0.0050
10	0	24	2.98	0.0065	3.05	0.0055
100	0	28	0.96	0.35	0.96	0.35
0	0.1	24	-2.23	0.036	-2.27	0.033
10	0.1	24	0.72	0.48	0.73	0.47
0	1	28	1.13	0.27	1.13	0.27
10	1	24	-0.97 (-3.55)	0.34 (0.002)	-1.04 (-3.40)	0.31 (0.002)

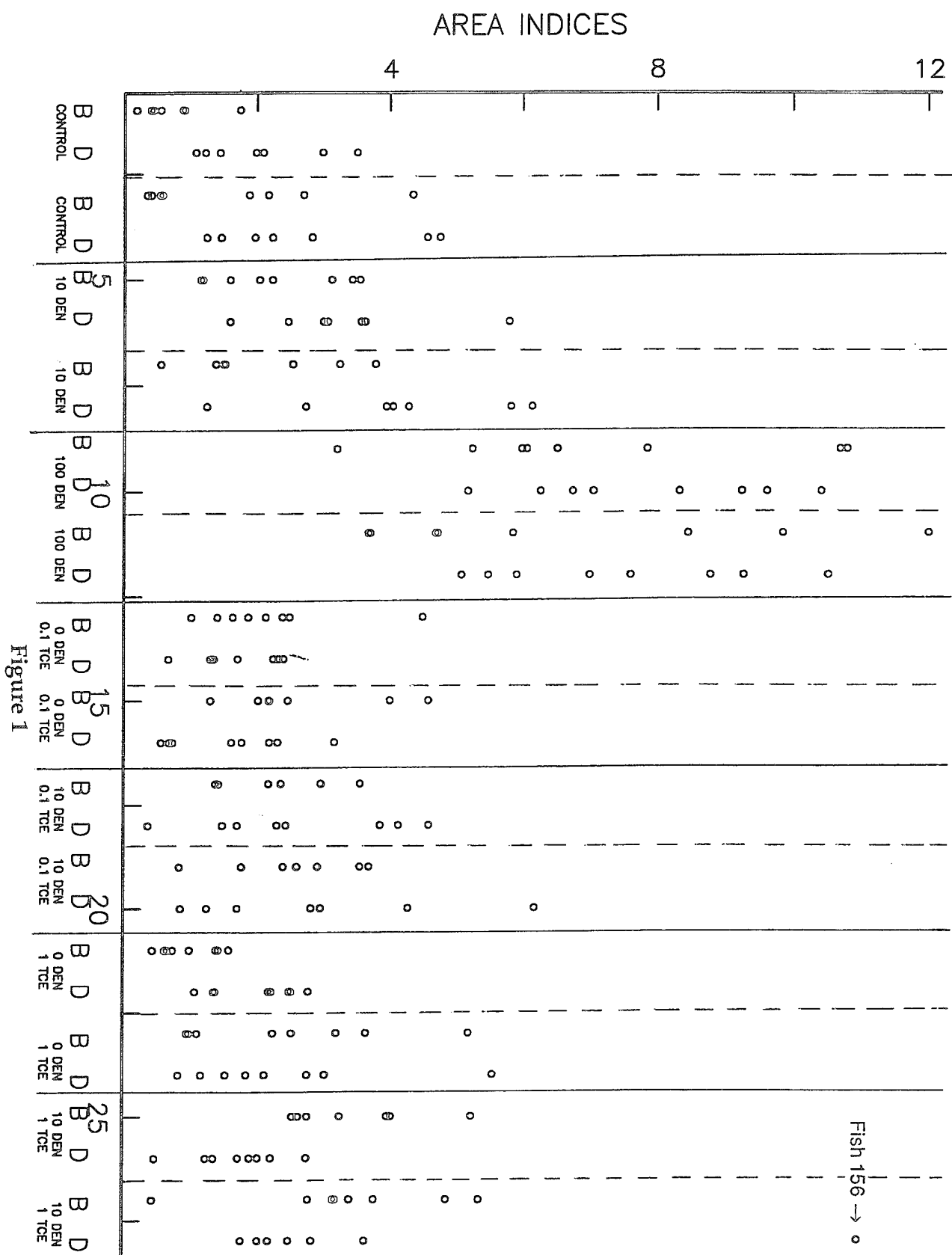
TABLE 3
Differences of Mean Area Indices Between Sacrifices B and D for Each
Treatment Group Minus the Mean Difference for the Control
with Fish 156
(without Fish 156)

Treatment		Approximate Degrees of Freedom	Different Variance Mean $t = \frac{\text{Mean}}{\text{Std. Error}}$	<i>p</i> -value $P\{ T > t\}$	Common Variance Mean $t = \frac{\text{Mean}}{\text{Std. Error}}$	<i>p</i> -value $P\{ T > t\}$
DEN	TCE					
10	0	48	0.29	0.77	0.34	0.74
100	0	48	-0.43	0.67	-0.49	0.63
0	0.1	48	-3.75	0.005	-4.39	0.00006
10	0.1	48	-1.37	0.18	-1.60	0.12
0	1	48	-1.38	0.17	-1.61	0.11
10	1	48	-2.36 (-4.62)	0.02 (0.00003)	-2.87 (-5.24)	0.006 3.5×10^{-6}

TABLE 4
Differences of Mean Area Indices Between Sacrifices B and D
By Tank
with Fish 156
(without Fish 156)

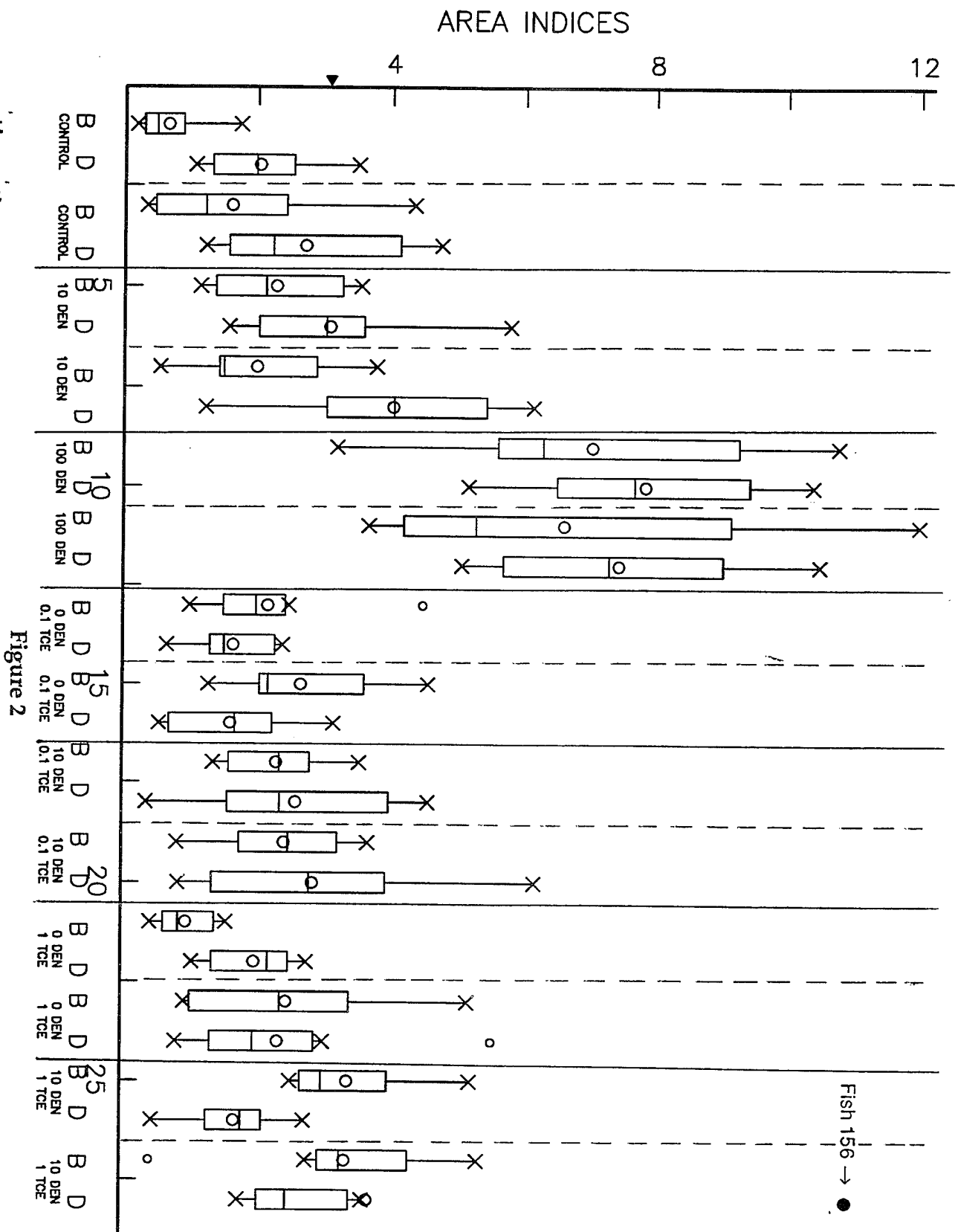
Tank	Treatment		Approximate Degrees of Freedom	Different Variance Mean $t = \frac{\text{Mean}}{\text{Std. Error}}$	$p\text{-value}$ $P\{ T > t\}$	Common Variance Mean $t = \frac{\text{Mean}}{\text{Std. Error}}$	$p\text{-value}$ $P\{ T > t\}$
1	0	0	14	3.92	0.002	3.92	0.002
2	0	0	12	1.49	0.16	1.55	0.15
3	10	0	14	1.36	0.20	1.36	0.20
4	10	0	12	2.72	0.02	2.91	0.01
5	100	0	14	0.72	0.48	0.72	0.48
6	100	0	14	0.64	0.53	0.64	0.53
7	0	0.1	14	-1.18	0.26	-1.18	0.26
8	0	0.1	12	-1.90	0.08	-1.94	0.06
9	10	0.1	12	0.49	0.63	0.47	0.65
10	10	0.1	12	0.54	0.60	0.58	0.57
11	0	1	14	3.74	0.0022	3.74	0.0022
12	0	1	14	-0.17	0.87	-0.17	0.87
13	10	1	14	-4.07	0.001	-4.07	0.001
14	10	1	12	0.25 (-1.49)	0.81 (0.17)	0.27 (-1.45)	0.79 (0.18)

AREA INDICES BY TANK



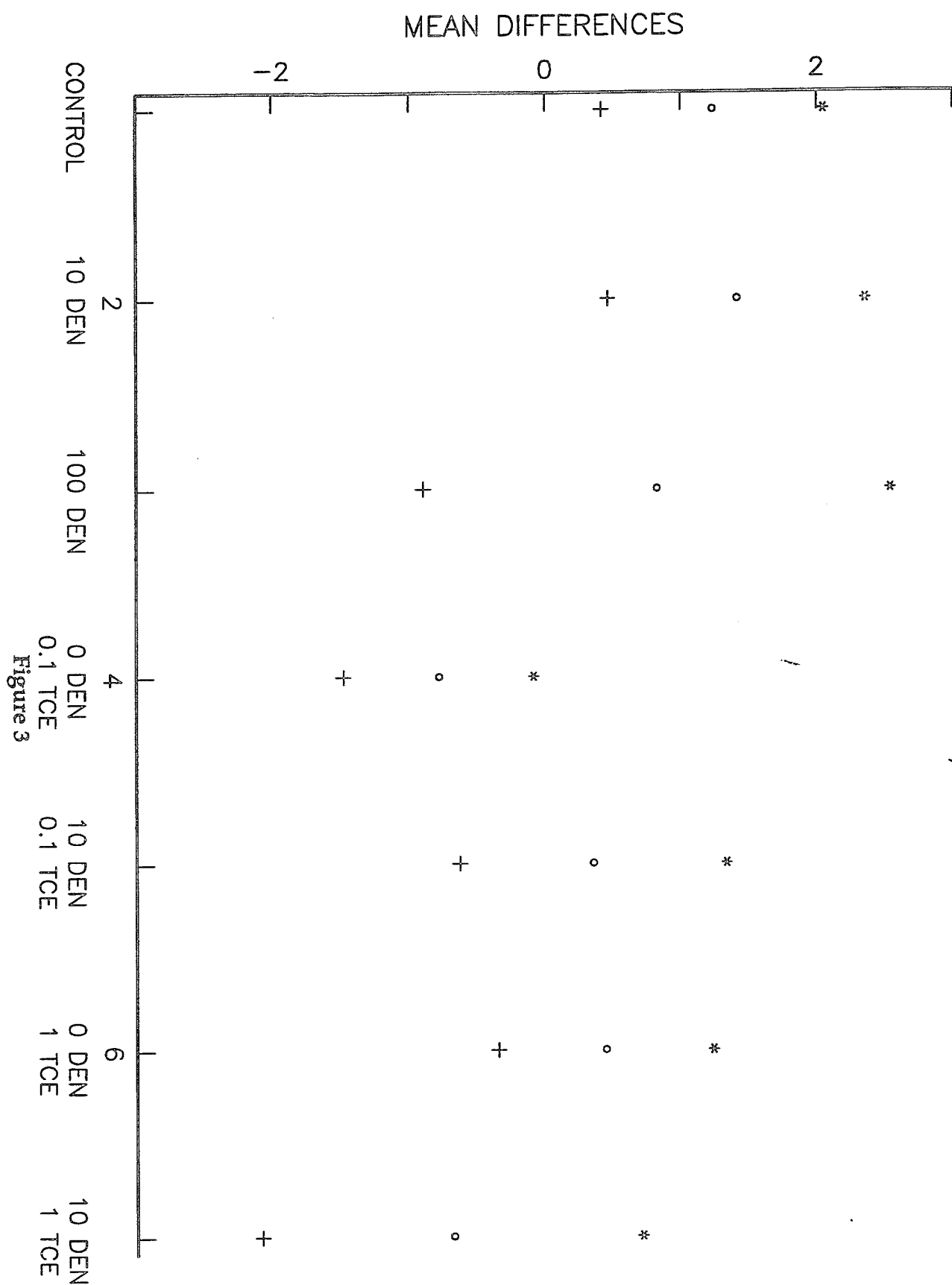
AREA INDICES BY TANK

BOX/PERCENTILE PLOTS, SSZ=218



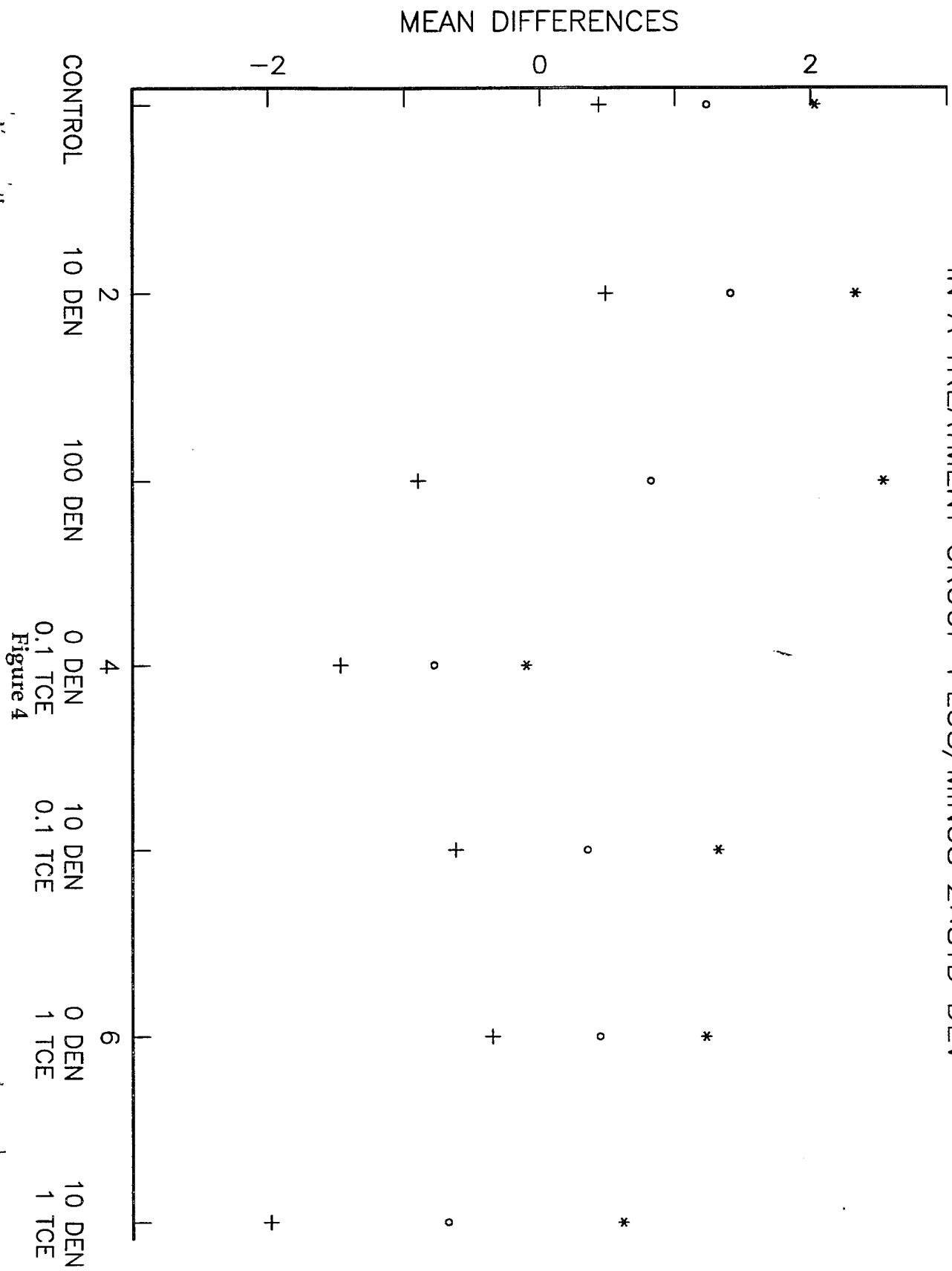
DIFFERENT TANK VARIANCES WITH FISH 156

AVERAGE OF MEAN AREA INDEX SACR D-SACR B FOR BOTH TANKS
IN A TREATMENT GROUP PLUS/MINUS 2xSTD DEV



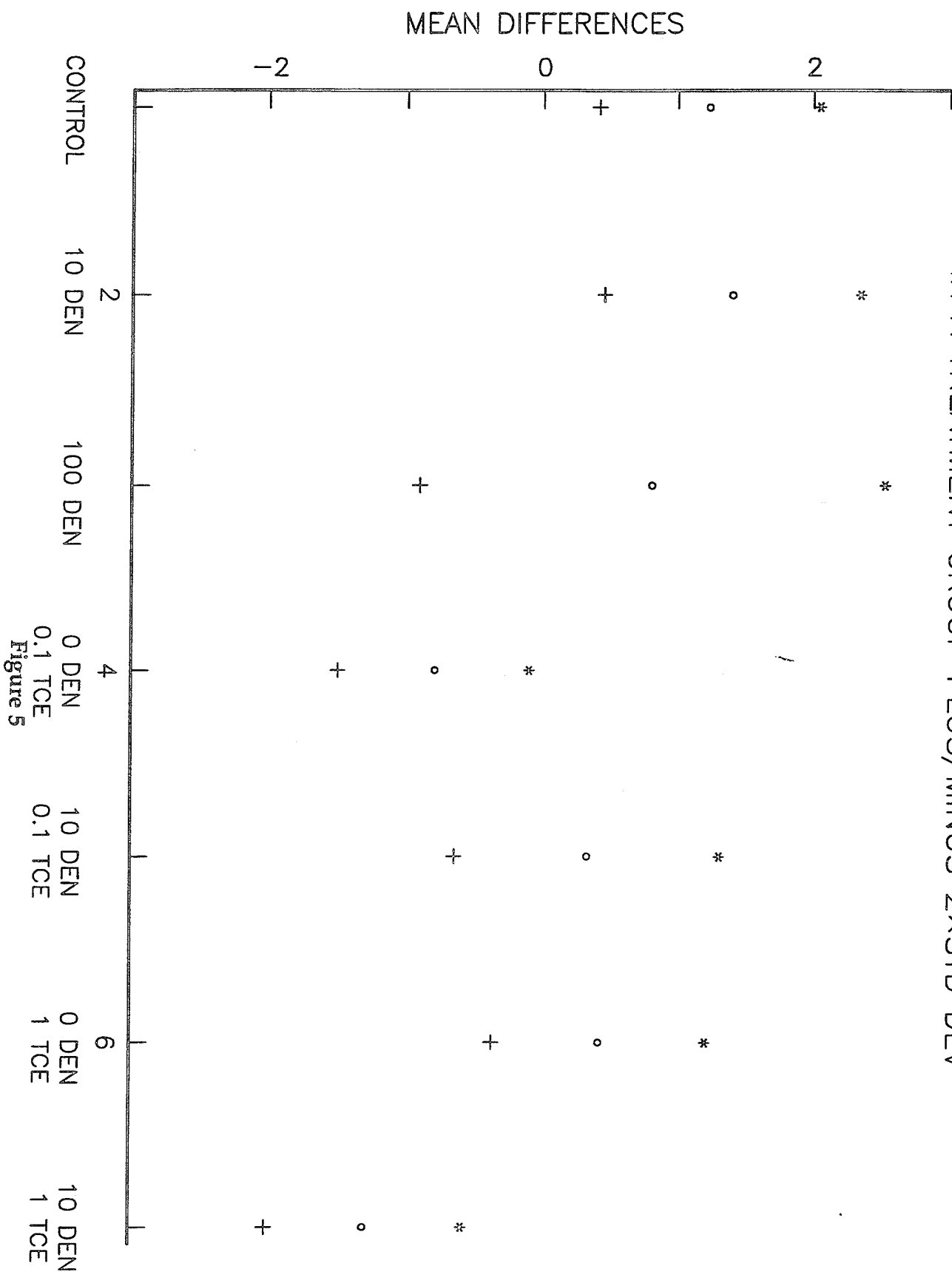
EQUAL TANK VARIANCES WITH FISH 156

AVERAGE OF MEAN AREA INDEX SACR D-SACR B FOR BOTH TANKS
IN A TREATMENT GROUP PLUS/MINUS 2xSTD DEV



DIFFERENT TANK VARIANCES WITHOUT FISH 156

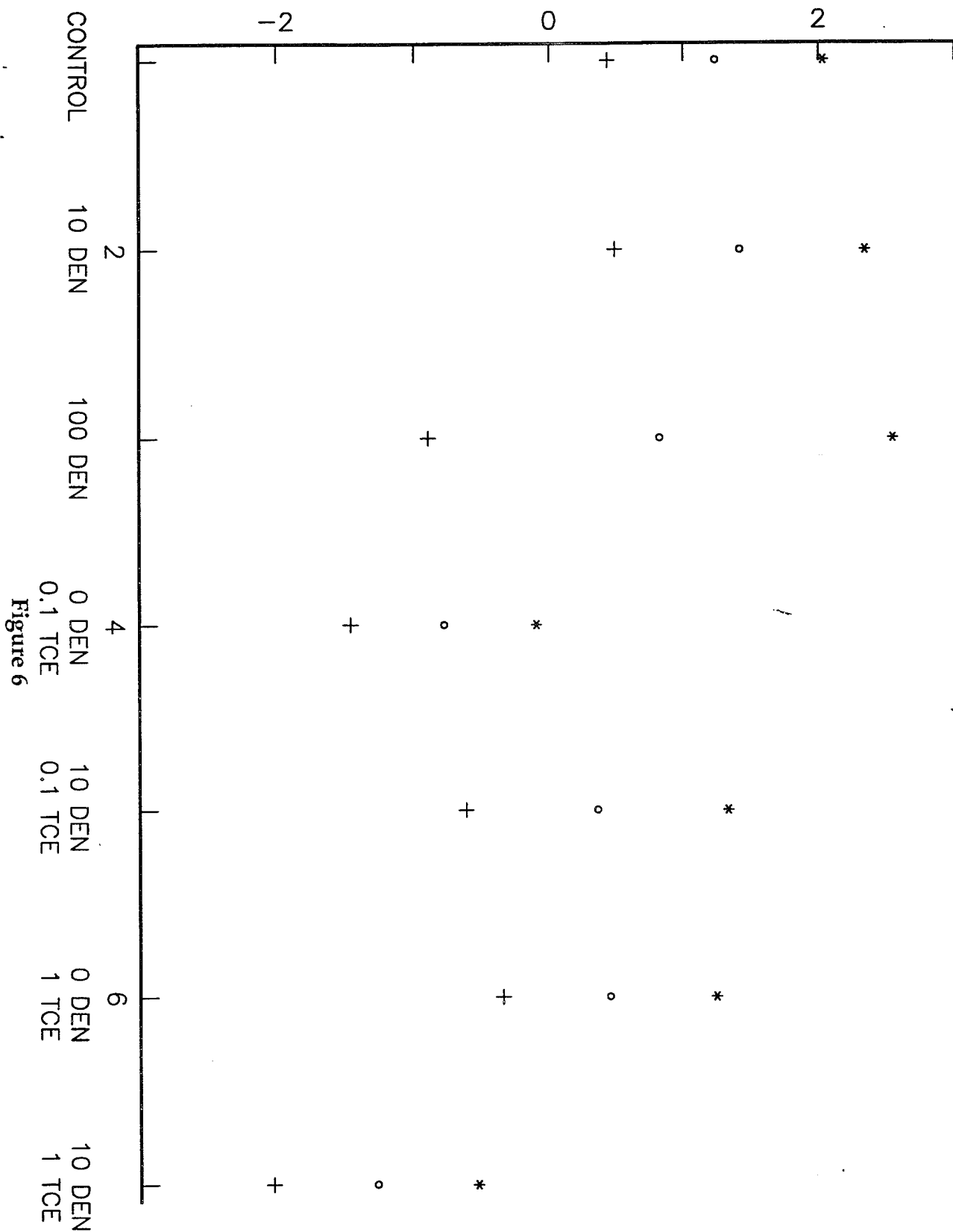
AVERAGE OF MEAN AREA INDEX SACR D-SACR B FOR BOTH TANKS
IN A TREATMENT GROUP PLUS/MINUS 2XSTD DEV



EQUAL VARIANCES WITHOUT FISH 156

AVERAGE OF MEAN AREA INDICES SACR D-SACR B FOR BOTH TANKS
IN A TREATMENT GROUP PLUS/MINUS 2XSTD DEV

MEAN DIFFERENCES



APPENDIX 4

Modeling and Statistical Analysis of Bioassay Data: Medaka Cell Proliferation under DEN and TCE

by

Donald P. Gaver

and

Patricia A. Jacobs

Department of Operations Research
Naval Postgraduate School
Monterey, CA 93943

1. INTRODUCTION

This report describes an analysis of cell proliferation data, by liver slice, from an experiment using Japanese Medaka. Previous work has used summary data from the same experiment (Gaver and Jacobs, 1994a,b). Another relevant reference is to Morris (1993). A brief description of the experiment follows.

The medaka are exposed to differing levels of DEN and TCE in tanks of water. The treatment groups are: control, 10 mg/ ℓ DEN, 100 mg/ ℓ DEN, 0.1 mg/ ℓ TCE, (10 mg/ ℓ DEN with 0.1 mg/ ℓ TCE), 1 mg/ ℓ TCE, and (10 mg/ ℓ DEN with 1 mg/ ℓ TCE). Each treatment group has two replicate tanks. Eight animals in each tank were sacrificed on 4 August, 1993; this is sacrifice B. Eight additional animals in each tank were sacrificed on 20 August 1993; this is sacrifice D.

Each sacrificed fish was exposed to BrdU for 72 hours prior to sacrifice; any cell that is in *S*-phase during this time has a BrdU marker. Each sacrificed fish is frozen and sliced longitudinally into 7-micron sections. A third of the slices are stained with another agent. This agent stains nuclei with the BrdU marker black; these nuclei are called *positive*. It is 5 of the latter stained slices that are analyzed subsequently.

Five slices containing a portion of the liver are considered for each fish. A region of interest (ROI) is marked on the slice; the ROI is chosen to attempt to maximize the number of hepatocytes and minimize the number of nonhepatocytes present. The area of all of the hepatocytes within the region of interest is measured, and the area of positive nuclei within the region of interest is measured. The number of hepatocytes in the ROI, and the number of positive hepatocytes in the ROI were also counted for half the fish in sacrifice B.

A count measure of cell proliferation, the *count index* (CI), for a slice is the number of positive hepatocytes in the ROI divided by the number of hepatocytes in the ROI, multiplied by 100. Evaluation of this measure is very labor intensive. As an alternative, the following *area index* (AI) is used

$$AI = \text{Area Index} = \frac{\text{Area of positive nuclei in the ROI}}{\text{Area of hepatocytes in the ROI}} \times 100.$$

The area index is easier to obtain; however, it does not quantify cells in *S*-phase exactly as does the CI since cells are of different size, as are the areas resulting from the slicing process, which are the result of a random intersection with the cell.

Figure 1 displays a plot of the count index (divided by 100) versus area index (divided by 100), computed by slice, for those fish of sacrifice B for which both measures are available, along with a simple unweighted least-squares-fitted

straight line; also displayed is the least-squares line equation with the standard errors of the coefficients displayed in parentheses below the coefficients. There appears to be a satisfactory linear relationship between the count index and area index, indicating that AI and CI are generally measuring the same response. However the variability of the area index increases as the count index increases; this increase is generally associated with high DEN and TCE concentration levels; its biological interpretation is not yet available. It suggests that cell sizes become more variable under concentration.

Figure 2 is a display of the slice area indices (divided by 100), by fish, for the two control tanks. Note the variability between fish and the somewhat greater variability between fish in tank 2 as compared to those in tank 1.

Several slice area datum appear to be missing or are of doubtful validity. These have been deleted from analytical consideration. They are listed in Table 1. An alternative might have been to use robust statistical procedures throughout; such procedures automatically down-weight highly discrepant observations. Furthermore, examination of the weights indicates discrepancy so that explanations can be sought. It seems likely that robust methods should be more widely used in environmental toxicology. Robust statistical methods are discussed seriously in Cox and Hinkley (1974). A less advanced treatment appears in Koopmann (1987).

A summary of the findings of the data analysis is as follows.

- a. Available data from sacrifice B suggests that there is a reasonably strong linear association between the count index and the area index. For ease of analysis the area index has been used throughout.

TABLE 1
Slices Not Considered in the Data Analysis

MISSING SLICES: SACRIFICE B				
Missing Measurements are left blank.				
ID	No. of Slices	Tank	Treatment	Reason
9273802	All	8	0.1 TCE	Blank field
9273811	All	9	10 DEN, 0.1 TCE	Blank field
MISSING SLICES: SACRIFICE D				
Missing Values appear to be coded with 0.				
ID	No. of Slices	Tank	Treatment	Reason
9273986	All	2	Control	Area ROI = Positive Area = 0
9285049	All	10	1 TCE	Area ROI = Positive Area = 0
9274001	All	4	10 DEN	All positive areas = 0 with 2 slices having 1 mit. hep.
9285079	All	14	10 DEN, 1 TCE	All positive areas = 0 with 3 slices having 1 mit. hep.
9285022	1	7	0.1 TCE	Area of ROI = 247.16, others of order 7500
9285043	1	9	10 DEN, 0.1 TCE	Positive area of slice = 0; 0 mit. cells
9285073	1	13	10 DEN, 1 TCE	Positive area of slice = 0; 0 mit. cells

- b. There is evidence that the variances of the slice area indices over fish exposed to various treatments are approximately equal to the corresponding means. This relationship can lead to misleading conclusions if standard statistical procedures are used uncritically; furthermore, the results are less efficient than necessary. However, the variances of the *square roots* of the slice area

indices for fish subjected to the various treatment (DEN and TCE) levels appear approximately constant, i.e. far less dependent on the corresponding mean values. Consequently, the square roots of the area indices are used in subsequent data analyses. The underlying reason for the above data behavior is that positive counts are rare random events, hence tend to be approximately Poisson distributed. The square root transformation is known to stabilize the variance of such counts; see Miller (1986), p. 59. The same transformation should, and here does, stabilize the variance of count-associated areas.

- c. The means of the fish mean square root of area indices are considered for both sacrifices. There is generally more treatment effect for sacrifice B than for sacrifice D: examine p -values in lines 3 of Tables 2 and 3 for the overall analysis of variance indications to see that sacrifice D p -values are always larger than those for sacrifice B. Four out of five of the treatment means are significantly larger (at the 95% level) than that for the control for sacrifice B. There is no significant difference (95% level) between the treatment means and the control mean for sacrifice D. For sacrifice B, the treatment means for two out of the three levels of TCE with 10 mg/ ℓ DEN are significantly larger than those without DEN for sacrifice B; there is no significant difference for sacrifice D.
- d. The control mean for sacrifice D is significantly larger than the control mean for sacrifice B. The other treatment means for sacrifice D are not significantly different than those for sacrifice B.

- e. There is some suggestion that in the later sacrifice D, the presence of TCE *lowers* the mean of the area index. The biological mechanisms likely to explain this behavior are not yet available.

The above results are from one-way analysis of variance (ANOVA) of the square roots of the area indices, augmented by *multiple comparison* methods; the latter allow all possible pairwise comparisons to be made with a specified experiment-wise error rate, here 5%. Two multiple comparison methods were used: simultaneous confidence intervals using Studentized range distribution and Studentized maximum modulus confidence intervals.

Brief Overall Summary of Findings to Date

The above can be briefly, and simplistically, summarized as follows. While it can be said that there is a statistically significant difference between mean responses ($\sqrt{\text{AI}}$) to the various treatments with DEN and TCE, no simple and interpretable dose-response patterns have been found. In particular, response does not appear to increase (or decrease) systematically with dose increase, where "dose" includes time of exposure as well as increases in chemical concentration levels. It remains to be seen whether the latter inconclusivity is lessened by the analysis of more data (later sacrifices), by finding that experimental problems or biases occurred, *or*, more exciting, that the dose-responses observed can be explained by biological mechanism, and that the findings essentially reappear when further experiments and data analyses are conducted.

Section 2 presents results of graphical displays of the data. Section 3 presents results of exploratory analyses of variance. Results of exploratory linear

regression models are presented in Section 4. Multiple comparison results appear in Section 5.

2. GRAPHICAL SUMMARIES OF THE SLICE AREA INDICES

Figure 3a (respectively 3b) displays boxplots of the slice area indices by tank for sacrifice B, (respectively sacrifice D). The upper side of the box is at the 75% quantile of the tank area indices. The lower side of the box is at the 25% quantile of the tank area indices. The circles are the means and the middle bar is the median. The boxplots may be viewed as a graphical one-way analysis of variance. There is some tank effect within a treatment group. The greatest dose-response effect is clearly for the 100 DEN treatment.

Figures 4a and 4b display plots of the mean of the area indices for each tank versus the variance of the area indices for each tank. Also displayed is a 45° line. There appears to be a linear relationship between the mean and variance; in fact, the variances appear to be approximately equal to the means. This relationship between the means and variances may lead to misleading results if analysis of variance techniques are applied directly to the slice area indices; Miller (1986) and Box (1954) discuss the effects of inequality of variance on one-way analysis of variance. It is believed that such effects may well appear often in environmental toxicology, particularly where *counts*, or count-like phenomena, are found.

As noted earlier, one standard transformation that can be applied to data with variances approximately equal to means to attempt to make the variance of the transformed data more nearly constant is the *square root transformation*; see Miller (1986). Figures 5a and 5b display plots of the mean of the square root of the area indices for each tank versus the variance of the square root of the area indices for each tank. The variances now appear unrelated to the corresponding means.

Figures 6a and 6b display boxplots of the square roots of the slice area indices by tank. Note that the lengths (heights between quartiles) of the boxes are less variable than are those for the boxplots of the raw area indices themselves. In the remainder of this paper the square root of area indices will be used.

3. EXPLORATORY ANALYSES OF VARIANCE

Results of exploratory analyses of variances appear in Tables 2 – 4. The basic data are summaries of area indices and the square roots of area indices for each fish. Since the boxplots indicate that the 100 DEN treatment is associated with much larger area indices, the analyses of variances were done with and without 100 DEN. Table 2 shows the results by tank. Recall that small p -values will indicate tank, hence treatment, effect. The first row of the table indicates that the tank means of the fish mean area index are significantly different. However the second row of the table indicates that the tank means of the logarithms of the variance (log variance) of area indices for each fish are also significantly different.

TABLE 2
 p -Values for Exploratory ANOVA of Area Indices (AI) by Tank
(AI = [(Positive Area)/ROI Area] \times 100)
(Small p -Values Indicate Chemical-Tank Effect)

Data	Sacrifice B		Sacrifice D	
	with 100 DEN	without 100 DEN	with 100 DEN	without 100 DEN
Mean slice AI for each fish in a tank	$< 10^{-16}$	3×10^{-5}	6×10^{-16}	2.9×10^{-2}
Log variance of slice AI for each fish in a tank	4×10^{-11}	2×10^{-5}	3×10^{-2}	0.54
Mean slice $\sqrt{\text{AI}}$ for each fish in a tank	4×10^{-16}	8×10^{-7}	1×10^{-14}	1.6×10^{-2}
Log variance of slice $\sqrt{\text{AI}}$ for each fish in a tank	0.16	0.25	0.89	0.97

The graphical analysis has already indicated this difference. Now recall that one of the assumptions of analysis of variance is that the data come from populations with equal variance, so apply the square-root transformation. Rows 3 and 4 report results using the square roots of the area indices. Note that the results of row 4 indicate that there is no significant difference between the tank means of the log variance of the slice square root of area index for each fish. However, the results of row 3 indicate that there *is* significant difference between the tank *means* of the mean of the slice square root area indices for each fish; note that the *p*-values for the tank means are smaller using the fish mean square root of area indices rather than the raw (untransformed) area indices for the analysis of variance (without the 100 DEN treatment). Thus, the difference in variances of the raw (untransformed) area indices appears to have masked some of the difference in means, presumably resulting from treatment effects.

Table 3 displays results for analyses of variance, with the two tanks in each treatment combined. Once again, the analysis of variance using the mean slice square root of the area indices for each fish indicates that there is a significant difference between treatment means of the means, even without 100 DEN.

Table 4 displays results of an analysis of variance of the mean square root of area indices for each fish in a treatment but without the control. Once again there is evidence of significant differences between the treatment means.

We conclude that there is a definite treatment effect, i.e. response to different treatment levels, even if no treatment = control and the “strong” 100 DEN treatment responses are removed.

TABLE 3

***p*-Values for Exploratory ANOVA of Area Indices (AI) by Treatment**
(AI = [(Positive Area)/ROI Area] × 100)
Treatment: 2 Tanks Combined
(Small *p*-Values Indicate Treatment Effect)

Data	Sacrifice B		Sacrifice D	
	with 100 DEN	without 100 DEN	with 100 DEN	without 100 DEN
Mean slice AI for each fish in a treatment	1×10^{-16}	1×10^{-5}	1×10^{-16}	2×10^{-2}
Log variance of slice AI for each fish in a treatment	4×10^{-13}	3×10^{-6}	1×10^{-4}	0.33
Mean slice $\sqrt{\text{AI}}$ for each fish in a treatment	2×10^{-16}	9×10^{-7}	4×10^{-16}	9×10^{-3}
Log variance of slice $\sqrt{\text{AI}}$ for each fish in a treatment	0.19	0.46	0.56	0.89

TABLE 4

***p*-Values for Exploratory ANOVA of Area Indices (AI) by Treatment**
Without Control
(AI = [(Positive Area)/ROI Area] × 100)
Treatment: 2 Tanks Combined
(Small *p*-Values Indicate Treatment Effect)

Data	Sacrifice B		Sacrifice D	
	with 100 DEN	without 100 DEN	with 100 DEN	without 100 DEN
Mean $\sqrt{\text{AI}}$ for fish	0	1.9×10^{-3}	5.9×10^{-15}	7.4×10^{-3}

4. EXPLORATORY LINEAR REGRESSION

Tables 5 – 8 report results of fitting exploratory linear regression models to the square root of the slice area indices for each fish. For Tables 5 and 6, the covariates are the level of DEN minus its mean; the level of TCE minus its mean;

and an interaction term: {(level of DEN minus its mean) times (level of TCE minus its mean)}. The means were subtracted to give the interaction term a value other than 0 if the level of DEN or the level of TCE is 0. The linear regressions were fit with and without the 100 DEN treatment. The results of Table 5 are for sacrifice B and those of Table 6 are for sacrifice D.

In both tables the values of R^2 are small when the 100 DEN data are excluded. This implies that a linear function of the above explanatory variables does not explain the data well. However, the standard errors of the estimates are also small. This behavior suggests that, although there may be an association between levels of DEN and TCE and the square root of the area index, that association is not linear. Note that for sacrifice B all of the estimates of the coefficients are significantly positive, suggesting that increasing levels of DEN and TCE are

TABLE 5
Sacrifice B: \sqrt{AI}
Linear Regression Coefficient Estimates with Standard Error
and 95% Normal Confidence Intervals

WITHOUT 100 DEN					
CONSTANT	DEN- \overline{DEN}	TCE- \overline{TCE}	(DEN- \overline{DEN}) \times (TCE- \overline{TCE})	R^2	s.e.
(SE)	(SE)	(SE)	(SE)	—	—
[CI]	[CI]	[CI]	[CI]	—	—
1.88	0.037	0.526	0.025	0.15	0.50
(0.067)	(0.005)	(0.148)	(0.010)		
[1.75,2.01]	[0.028,0.046]	[0.237,0.816]	[0.005,0.045]		
$\overline{DEN} = 5.0 \quad \overline{TCE} = 0.372$					
WITH 100 DEN					
1.77	0.029	0.710	0.039	0.56	0.50
(0.040)	(0.003)	(0.117)	(0.008)		
[1.69,1.85]	[0.024,0.033]	[0.48,0.94]	[0.02,0.05]		
$\overline{DEN} = 18.81 \quad \overline{TCE} = 0.318$					

TABLE 6
Sacrifice D: \sqrt{AI}
Linear Regression Coefficient Estimates with Standard Error
and 95% Normal Confidence Intervals

<i>WITHOUT 100 DEN</i>					
CONSTANT	DEN- \overline{DEN}	TCE- \overline{TCE}	(DEN- \overline{DEN}) \times (TCE- \overline{TCE})	R ²	s.e.
(SE)	(SE)	(SE)	(SE)	—	—
[CI]	[CI]	[CI]	[CI]	—	—
1.89	0.028	-0.402	-0.025	0.08	0.50
(0.07)	(0.005)	(0.151)	(0.01)		
[1.76,2.03]	[0.02,0.04]	[-0.70,-0.11]	[-0.05,-0.005]		
$\overline{DEN} = 4.88 \quad \overline{TCE} = 0.37$					
<i>WITH 100 DEN</i>					
1.68	0.013	-0.050	0.0005	0.45	0.50
(0.04)	(0.003)	(0.12)	(0.008)		
[1.60,1.76]	[0.008,0.018]	[-0.29,0.19]	[-0.02,0.02]		
$\overline{DEN} = 19.05 \quad \overline{TCE} = 0.315$					

associated with higher area indices. However, for sacrifice D, the estimate of the coefficient of the level of TCE and the estimate of the coefficient of the interaction term coefficient are significantly negative for the regression, even without the 100 DEN treatment. This suggests that for the later sacrifice, exposure to TCE may have an inhibitory effect. The only estimate of covariate that is significantly different from 0 for sacrifice D when 100 DEN is included is exposure to DEN.

Tables 7 and 8 report results of fitting linear regressions with the covariate being the level of TCE exposure to data from fish not exposed to DEN and to data from fish exposed to 10 mg/ ℓ DEN; the dependent variable is the square root of the slice area index. The R^2 values are very small, indicating a lack of linear fit, but the estimate coefficients for TCE in the regressions using data from

fish exposed to 10 mg/ℓ DEN are formally significant. Once again, this behavior suggests that there may be an association but the association is not linear.

For sacrifice B, there was no significant effect for the level of TCE for the fish not exposed to DEN; for those fish exposed to 10 mg/ℓ DEN the coefficient for level of TCE is significantly positive indicating that increasing levels of TCE are associated with increasing (square roots of) area indices.

TABLE 7
Sacrifice B: \sqrt{AI}
Linear Regression Coefficient Estimates with Standard Error
and 95% Normal Confidence Intervals
(Replicate Tanks Pooled)

<i>NO DEN</i>				
CONSTANT (SE) [CI]	TCE (SE) [CI]	DEN (SE) [CI]	R ² — —	s.e. — —
1.18	0.056	—	0.002	0.55
(0.047)	(0.080)	—		
[1.09, 1.27]	[-0.10, 0.21]	—		
<i>10 DEN</i>				
1.47	0.31	—	0.09	0.44
(0.037)	(0.06)	—		
[1.40, 1.54]	[0.18, 0.43]	—		
<i>0 DEN and 10 DEN</i>				
1.14	0.18	0.038	0.14	0.50
(0.038)	(0.051)	(0.005)		
[1.06, 1.21]	[0.08, 0.28]	[0.29, 0.047]		

In sacrifice D, for those fish not exposed to DEN, the estimate of the coefficient of TCE is not significantly different than 0. However, for those fish exposed to 10 mg/ℓ DEN the estimate of the coefficient of TCE is significantly *negative*, suggesting that for the fish of the later sacrifice that were exposed to

DEN, the *greater* the level of TCE exposure, the *smaller* the (square root of) the area index. This effect calls for biological explanation.

TABLE 8
Sacrifice D: \sqrt{AI}
Linear Regression Coefficient Estimates with Standard Error
and 95% Normal Confidence Intervals
(Replicate Tanks Pooled)

<i>NO DEN</i>				
CONSTANT (SE) [CI]	TCE (SE) [CI]	DEN (SE) [CI]	R ² — —	s.e. — —
1.35	0.059	—	0.004	0.43
(0.037)	(0.062)	—		
[1.27, 1.42]	[-0.063, 0.181]	—		
<i>10 DEN</i>				
1.71	-0.189	—	0.02	0.56
(0.048)	(0.083)	—		
[1.61, 1.80]	[-0.352, -0.026]	—		
<i>0 DEN and 10 DEN</i>				
1.39	-0.061	0.027	0.07	0.50
(0.038)	(0.052)	(0.005)		
[1.32, 1.47]	[-0.16, 0.04]	[0.017, 0.036]		

5. MULTIPLE COMPARISONS

The exploratory analyses of variances strongly rejected the null hypothesis that all the treatment means (even without the 100 DEN treatment) of the fish mean square root of the area indices are equal. Rejection of the null hypothesis does not indicate specifically which means are not equal. A method for discovering which means differ is called a multiple comparisons procedure. There are a number of different *multiple comparisons* procedures in the literature; see Miller (1981). We will use two of them.

5.1 Simultaneous Confidence Intervals using Studentized Range Distribution

The first procedure uses the studentized range distribution to construct simultaneous confidence statements about the true values of all differences of the treatment means; this procedure constructs the Tukey (Studentized range distribution) simultaneous confidence intervals. Table 9 describes the procedure to obtain simultaneous 95% confidence intervals for all differences of treatment means for one sacrifice without the 100 DEN treatment. The original procedure requires that there be an equal number of fish in each treatment. However, Ott *et al.* suggest step 4 in Table 9 if the number of fish in each treatment do not differ by much.

5.1a Treatment Means Minus Control Mean

Figure 7 presents some of the 95% simultaneous confidence intervals of the differences of treatment means for sacrifice B. It shows the confidence intervals for the treatment means minus the control mean for the fish mean square root of the area indices. Note that 4 out of the 5 intervals are significantly above 0 indicating that the treatments are associated with a larger mean square root area indices than those for the control. The greatest difference is that for the treatment of 10 mg/ ℓ DEN with 1 mg/ ℓ TCE. However, since the confidence intervals overlap, there is no apparent association between the treatment and the magnitude of the differences in the means.

Figure 8 presents some of the 95% simultaneous confidence intervals for the later sacrifice D. It shows the confidence intervals for the treatment mean minus the control mean. Since the intervals include 0, none of the treatment means is significantly different from the control mean.

TABLE 9

To Obtain Tukey (Studentized Range Distribution) Simultaneous 95% Confidence Intervals for Treatment Mean Differences, One Sacrifice

1.	There are 6 treatments: Control; 10 DEN; 0.1 TCE; 10 DEN with 0.1 TCE; 1 TCE; and 10 DEN with 1 TCE.
2.	There are ~88 within degrees of freedom $\left(\sum_{i=1}^6 (n_i - 1) \right)$ where n_i is the number of fish in treatment i .
3.	The 0.05 percentage point for the studentized range for 60 within-degrees-of-freedom and 6 treatment means is 4.16, from published tables, <i>BIOMETRIKA Tables for Statisticians</i> , Vol. 1. This is larger than the percentage point for 88 within-degrees-of-freedom. Thus, the constructed confidence intervals will be conservative: one can truly say that <i>all</i> pairwise difference comparisons are made with (95%) confidence.
4.	Since the number of fish per treatment differs somewhat (due to missing fish) the harmonic mean of the number of fish per treatment is used <div style="text-align: center;"> $n = \frac{6}{\frac{1}{n_1} + \dots + \frac{1}{n_6}}$ </div> where n_i is the number of fish in treatment i .
5.	The mean square within is <div style="text-align: center;"> $\frac{\sum_{i=1}^6 \sum_j (y_{ij} - \bar{y}_i)^2}{\sum_{i=1}^6 (n_i - 1)} = \text{MS}(\text{within})$ </div> where y_{ij} is the mean $\sqrt{\text{AI}}$ for fish j in treatment i and \bar{y}_i is the mean of the mean $\sqrt{\text{AI}}$ for the fish in treatment i .
6.	95% confidence intervals for all pairs of means μ_i and $\mu_{i'}$ <div style="text-align: center;"> $(\bar{y}_i - \bar{y}_{i'}) \pm (4.16) \sqrt{\text{MS}(\text{within})/n}$ </div>

5.1b Treatment Means for Treatments with Exposure to 10 mg/ℓ DEN Minus Those without Exposure to 10 mg/ℓ DEN

Figure 9 displays the sacrifice B 95% simultaneous confidence intervals for the difference between the treatment means for mean fish square root of the area

indices for those treatments *with* 10 mg/ℓ DEN minus the treatment means for those treatments *without* 10 mg/ℓ DEN, by level of TCE exposure. Note that the treatment means with 10 mg/ℓ DEN is significantly larger than that without 10mg/ℓ DEN for 0 mg/ℓ TCE and 1 mg/ℓ TCE since the confidence intervals do not include 0. There is no significant difference for 0.1 mg/ℓ TCE since the confidence interval includes 0.

Figure 10 displays a similar plot for the later sacrifice D. There is no significant difference between the treatment means with 10 mg/ℓ DEN and those without.

5.1c Treatment Means for Sacrifice D Minus Treatment Means for Sacrifice B

Simultaneous confidence intervals are computed for all differences of the treatment means of the fish mean square root of the area indices for sacrifices B and D combined. Figure 11 displays six of the 95% simultaneous confidence intervals. It displays the 95% confidence intervals for the difference in treatment means between sacrifice D and sacrifice B. The only significant difference is for the control where the mean of the fish mean square root of area indices for sacrifice D is significantly larger than that for sacrifice B.

5.2 Studentized Maximum Modulus Confidence Intervals

Simultaneous confidence intervals for the treatment means themselves can be constructed using the studentized maximum modulus procedure; cf. Miller (1981). The procedure is as follows for the mean fish square root area index for the three treatment groups (10 mg/ℓ DEN, 0 mg/ℓ TCE), (10 mg/ℓ DEN, 0.1 mg/ℓ TCE), and (10 mg/ℓ DEN, 1 mg/ℓ TCE) for 1 sacrifice.

To Obtain Simultaneous Confidence Intervals for 3 Treatment Means using the Studentized Maximum Modulus Distribution

1. Compute the within degrees of freedom for the three treatments.

$$d = \sum_{i=1}^3 (n_i - 1)$$

where n_i is the number of fish in treatment i .

2. Compute the mean square within

$$MS(\text{within}) = \frac{\sum_{i=1}^3 \sum_j (y_{ij} - \bar{y}_i)^2}{\sum_{i=1}^3 (n_i - 1)}$$

where y_{ij} is the mean square root of the area indices for fish j in treatment i and \bar{y}_i is the mean of the fish means in treatment i .

3. Find the upper 0.05 point of the studentized maximum modulus distribution with parameters 3 (treatments) and d degrees of freedom, $m(3, d)$. Tables can be found in Miller (1981).
4. The three simultaneous 95% confidence intervals are

$$\bar{y}_i \pm m(3, d) \sqrt{MS(\text{within})/n_i}.$$

Figure 12 displays the 95% simultaneous confidence intervals for sacrifice B for the means of the fish mean of the square root of the slice area indices for those treatments having fish exposed to 10 mg/ℓ DEN. The means appear about the same for 0 mg/ℓ TCE and 0.1 mg/ℓ TCE. The mean for 1 mg/ℓ TCE appears to be somewhat larger.

Figure 13 displays the 95% simultaneous confidence intervals for sacrifice D for the means of those treatments with 10 mg/ ℓ DEN by level of TCE. There is some suggestion that the presence of TCE is associated with a *lower* mean of the fish mean square root of the slice area indices.

CONCLUSION

The above analyses illustrate the use of statistical methods appropriate for the kinds of data obtained by the medaka experiments. The methods of transformation, analysis of variance, and multiple comparisons are useful and powerful for the initial data analyses, suggesting some surprising dose-response relations that are worthy of careful further biological investigation and explanation. Alternative methods can also be applied, and should yield the same general insights.

REFERENCES

- Bickel, P. J. and Doksum, K. A. *Mathematical Statistics: Basic Ideas and Selected Topics*, Holden-Day, Inc., San Francisco, 1977.
- Box, G. E. P. "Some theorems on quadratic forms applied in the study of analysis of variance problems, I. Effect of inequality of variance in the one-way classification." *Annals of Mathematical Statistics*, **25**, pp. 290-302.
- Cox, D. R. and Hinkley, D. V. *Theoretical Statistics*, Chapman and Hall, New York, 1974.
- Gaver, D. P. and Jacobs, P. A. "Assessment of liver modification and cell proliferation in medaka under DEN and TCE dosage, using data available 4/26/94, suggestions for statistical analysis and results from exploratory data analysis." Working Paper, April 26, 1994.
- Gaver, D. P. and Jacobs, P. A. "Comparison of area indices in medaka livers for sacrifices at different dose time combinations using data available 5/19/94." Working Paper, May 19, 1994.
- Huff, J. "Absence of morphologic correlation between chemical toxicity and chemical carcinogenesis." *Environmental Health Perspectives*, **101** (Suppl. 5) pp. 45-54, 1993.
- IBM Corporation. A Graphical Statistical System (AGSS).
- Koopmann, L. H. *Introduction to Contemporary Statistical Methods*, Duxbury Press, Boston, 1987.
- Miller, R. G. Jr. *Beyond ANOVA, Basics of Applied Statistics*, John Wiley & Sons, New York, 1986.
- Miller, R. G. Jr. *Simultaneous Statistical Inference*, Second Edition, Springer-Verlag, New York, 1981.
- Morris, R. W. "Analysis of cell proliferation data." *Environmental Health Perspectives*, **101** (Suppl. 5) pp. 73-78, 1993.
- Ott, L. and Hildebrand, D. K. *Statistical Thinking for Managers*, PWS Publishers, Boston, 1983.

AREA INDEX VERSUS COUNT INDEX BY SLICE

SCATTER PLOT, SSZ=275

$$Y = 0.13857 + 39.226 \times X$$

(0.10) (1.04)

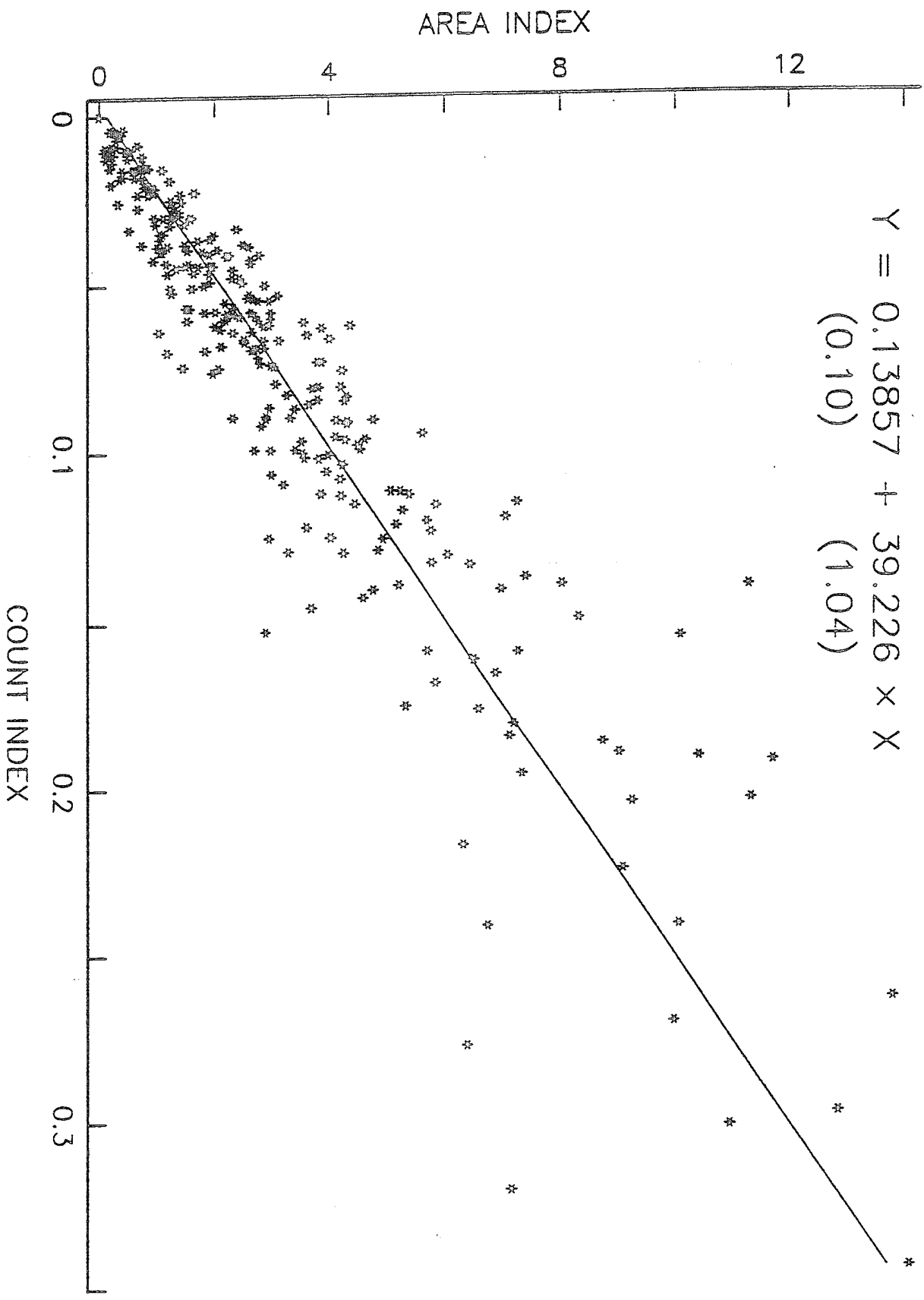
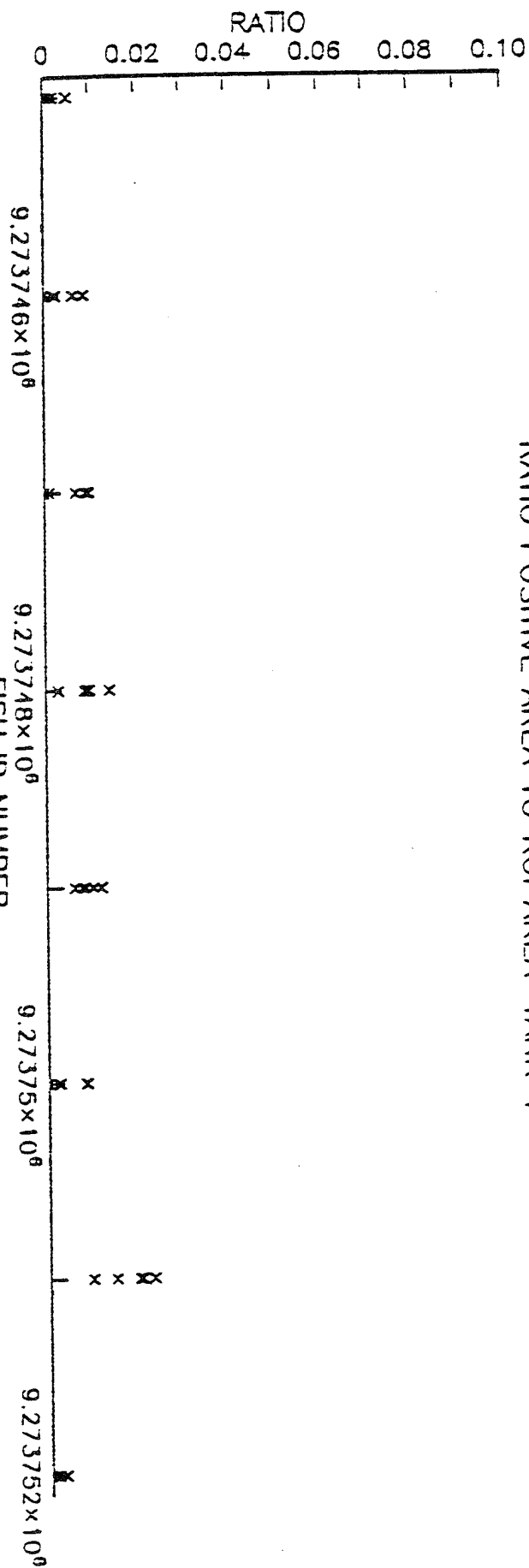


Figure 1

SACRIFICE B, CONTROL

RATIO POSITIVE AREA TO ROI AREA-TANK 1



RATIO POSITIVE AREA TO ROI AREA-TANK 2

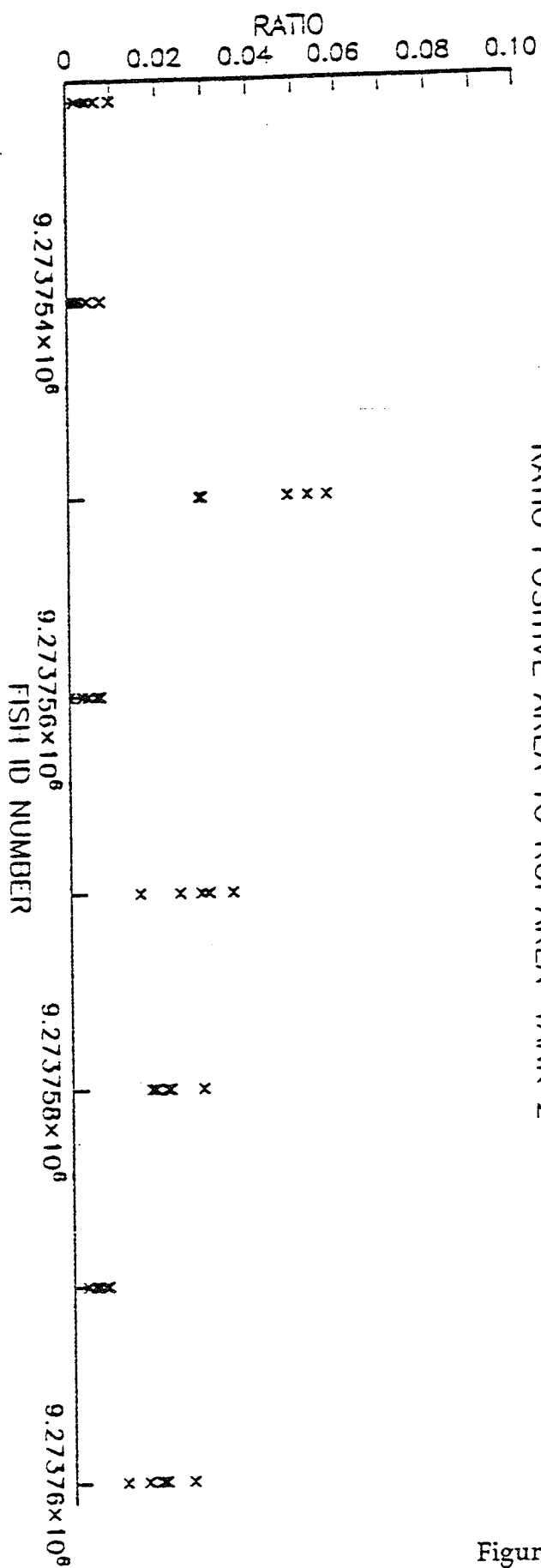


Figure 2

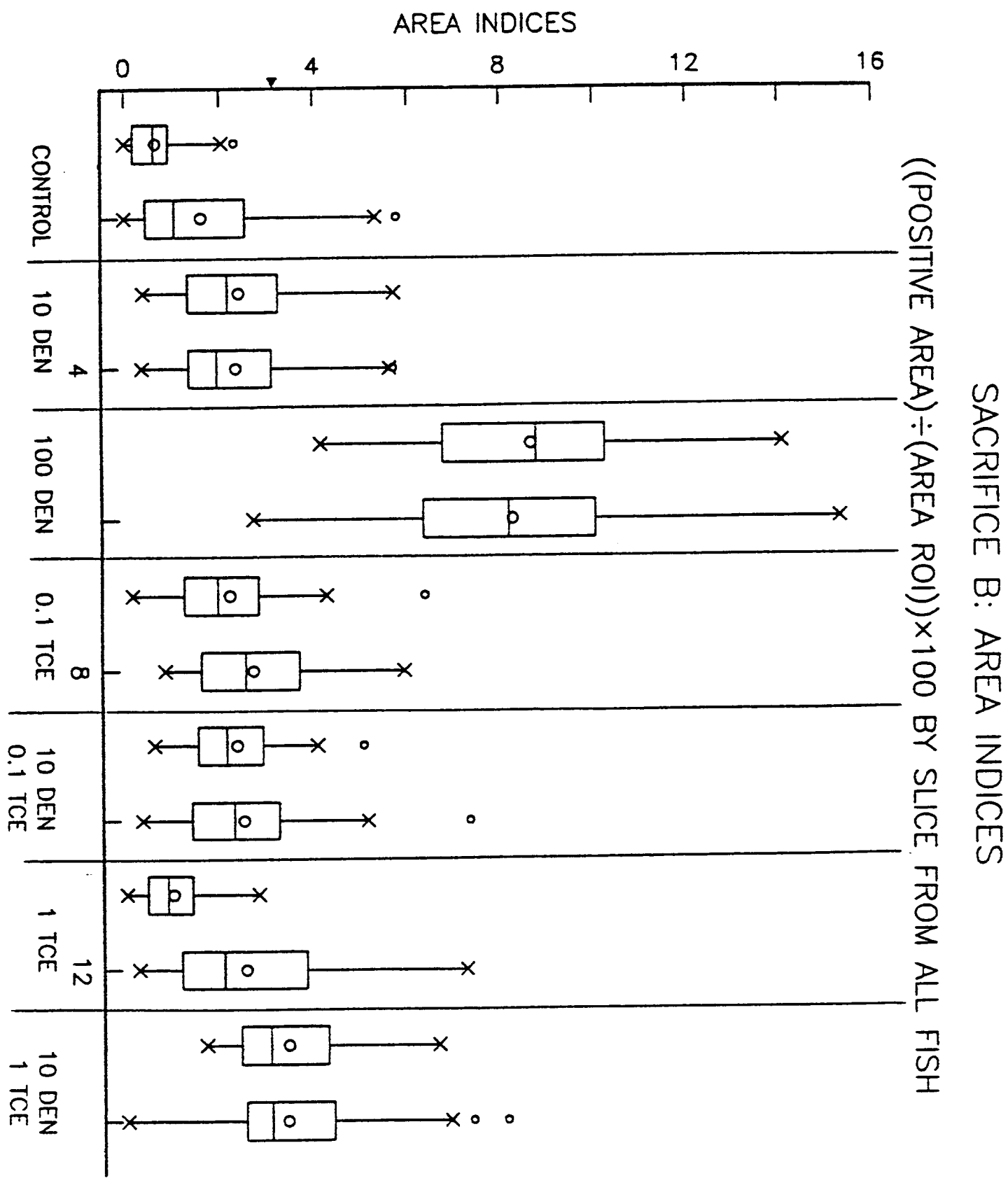


Figure 3a

AREA INDICES

SACRIFICE D: AREA INDICES

$((\text{POSITIVE AREA}) \div (\text{AREA ROI})) \times 100$ BY SLICE FROM ALL FISH

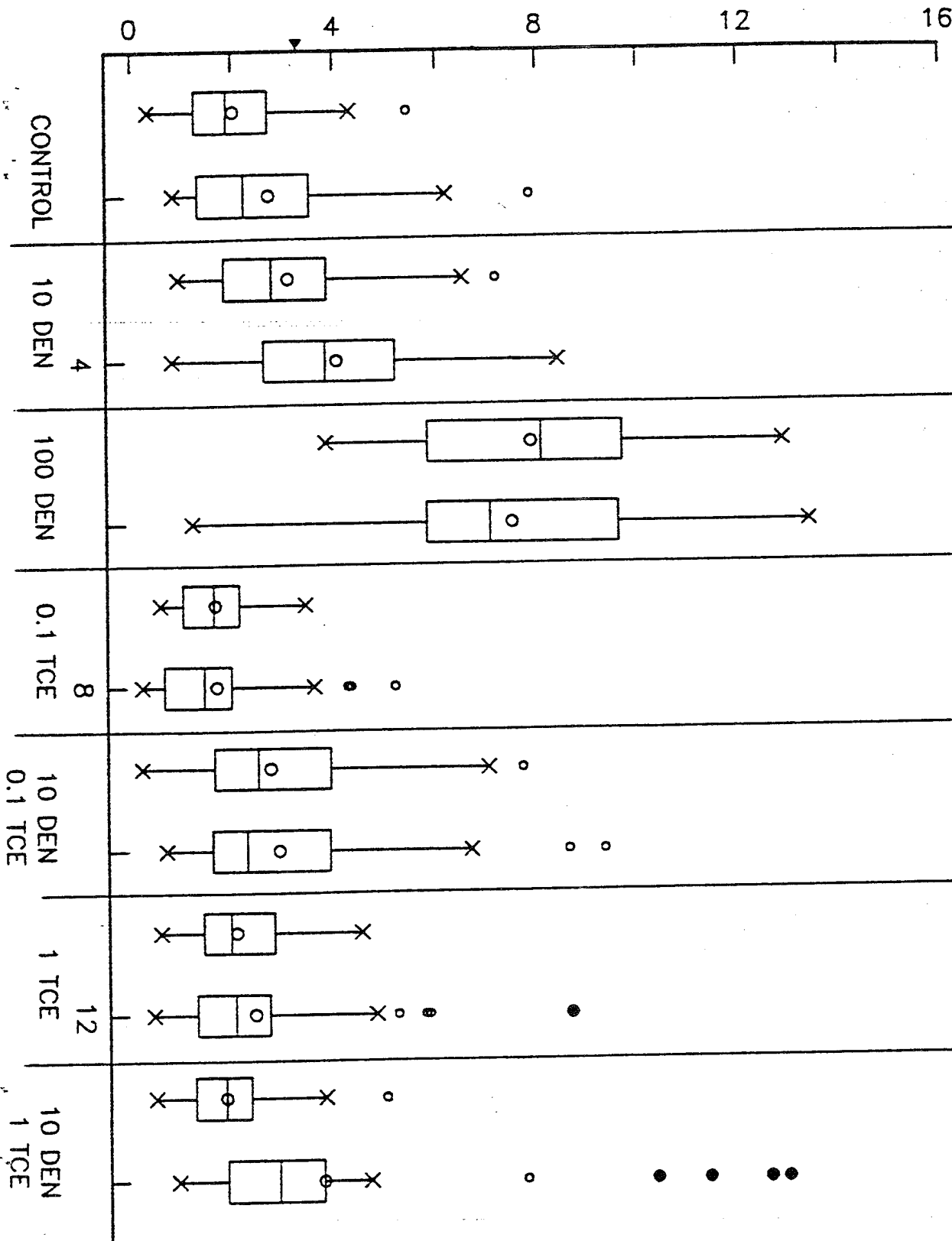


Figure 3b

SACRIFICE B: PLOT MEAN VS VARIANCE FOR EACH TANK
 VARIANCE OF RATIO OF (POSITIVE AREA ÷ AREA ROI) × 100
 USING SLICES WITHIN A TANK

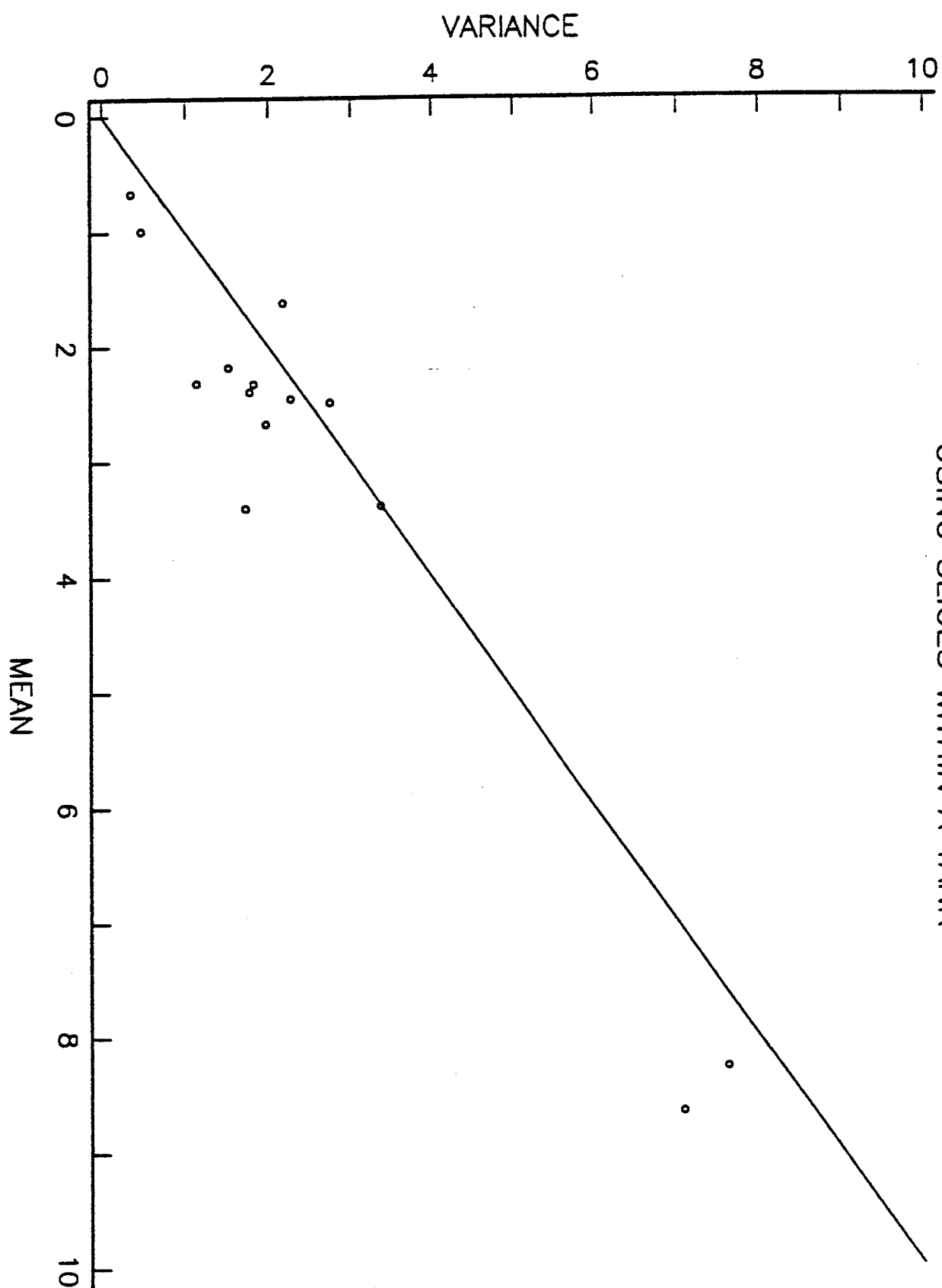


Figure 4a

SACRIFICE D: PLOT MEAN VS VARIANCE FOR EACH TANK
 VARIANCE OF RATIO OF (POSITIVE AREA ÷ AREA ROI) × 100
 USING SLICES WITHIN A TANK

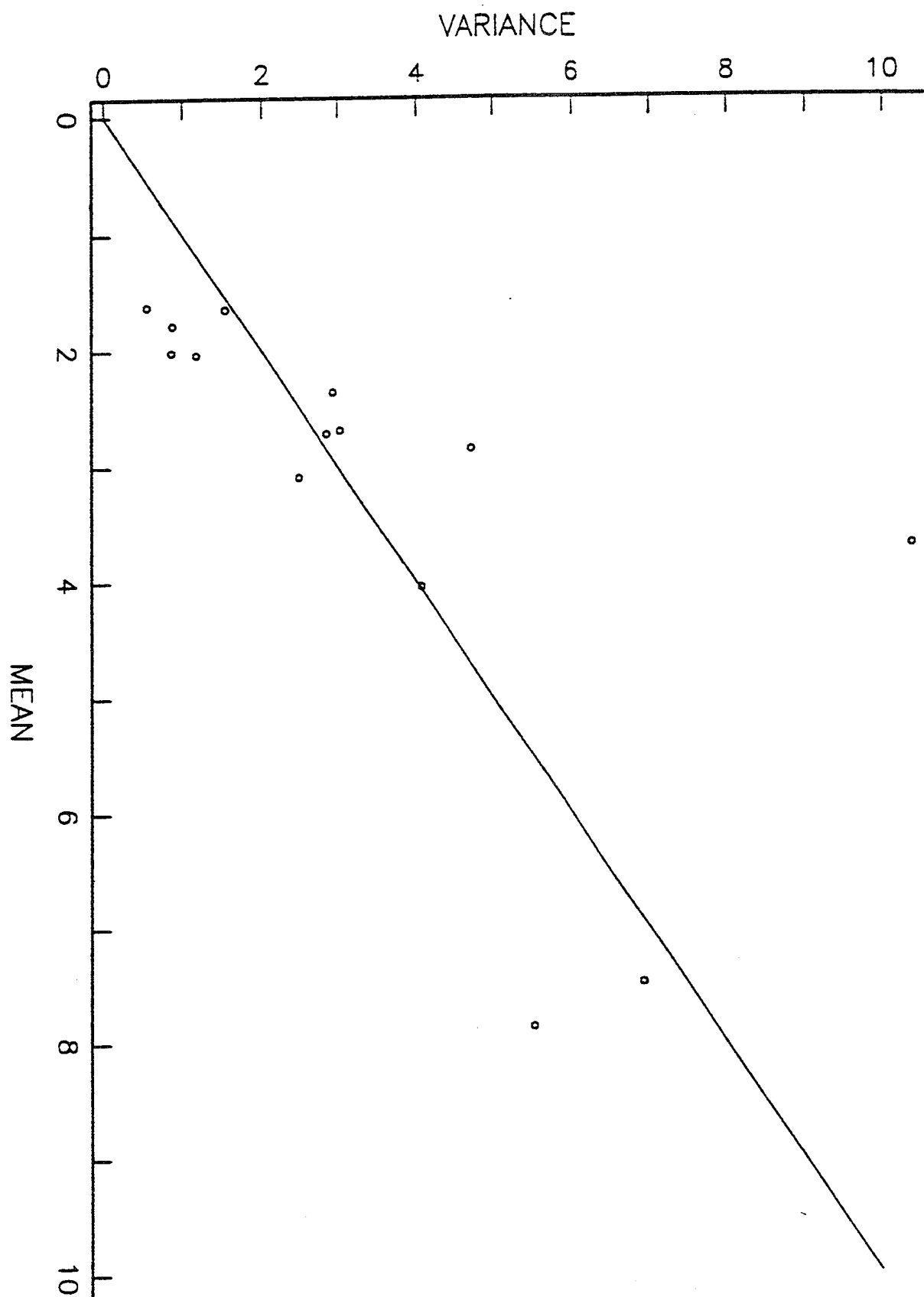


Figure 4b

SACRIFICE B: SQUARE ROOT AREA INDEX BY TANK

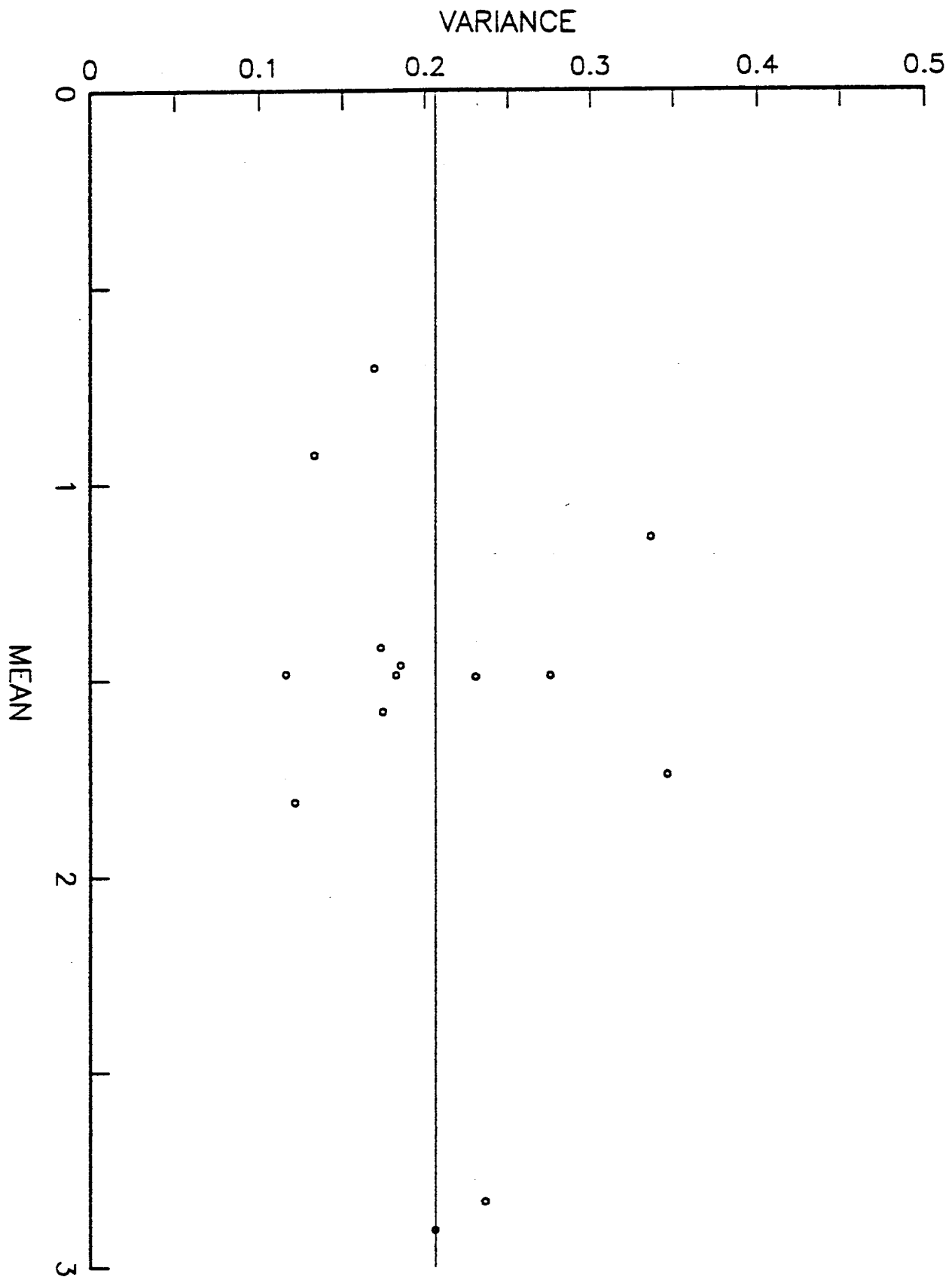


Figure 5a

SACRIFICE D: SQUARE ROOT AREA INDEX BY TANK

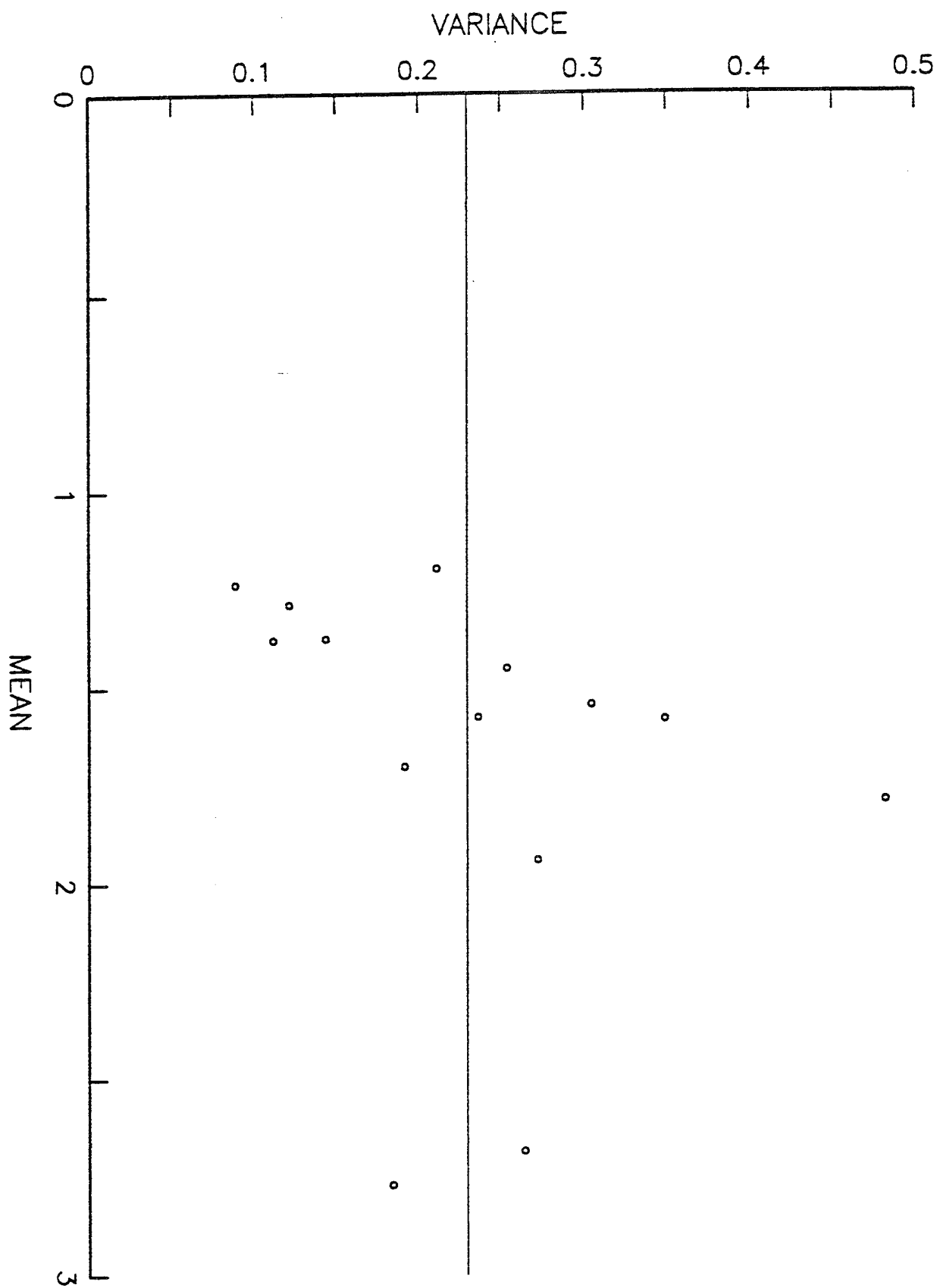


Figure 5b

SACRIFICE B

SQUARE ROOT AREA INDICES ((POSITIVE AREA)÷(AREA ROI))×100
BY SLICE FROM ALL FISH

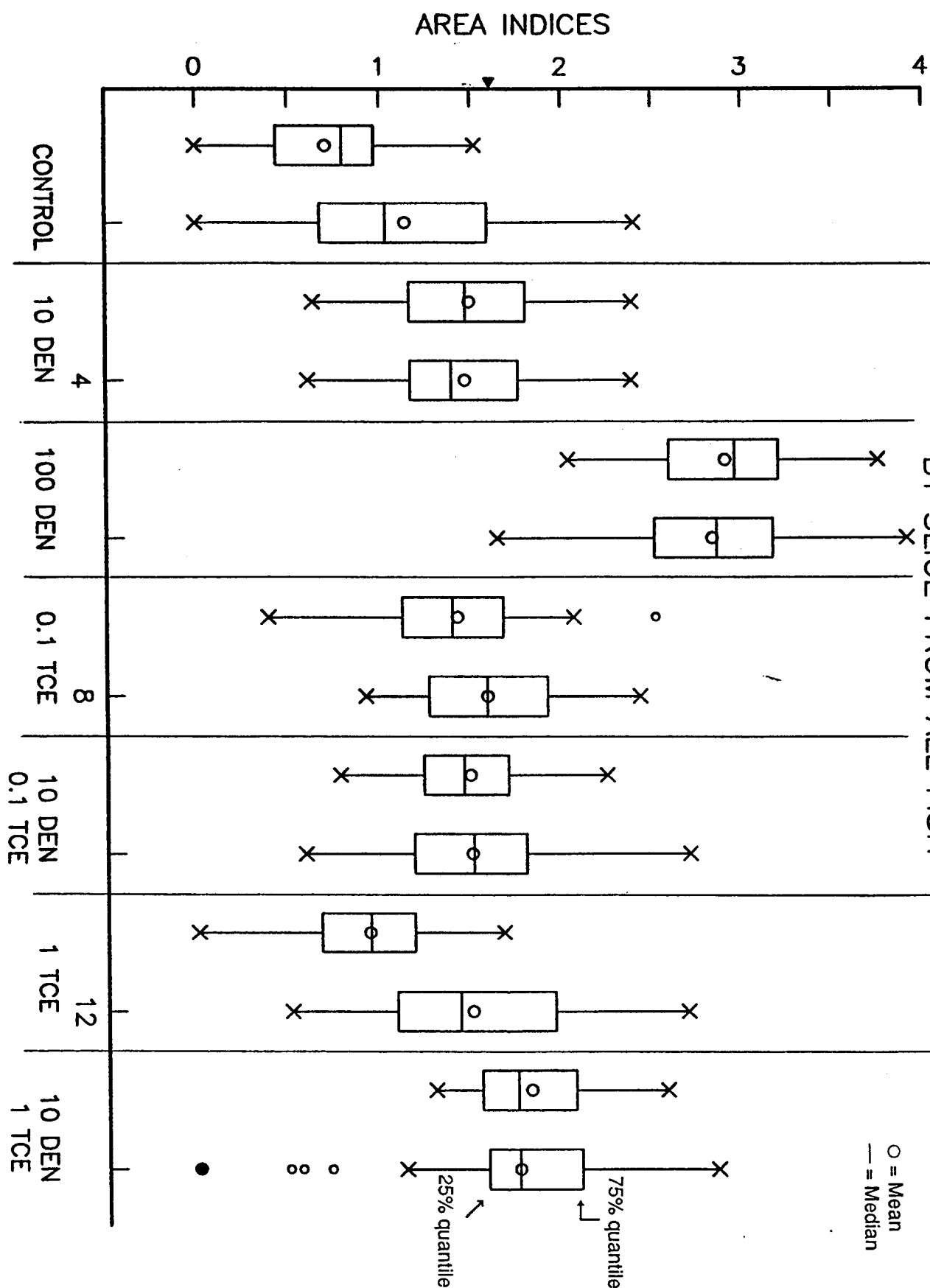


Figure 6a

SACRIFICE D

SQUARE ROOT AREA INDICES ((POSITIVE AREA)÷(AREA ROI))×100
BY SLICE FROM ALL FISH

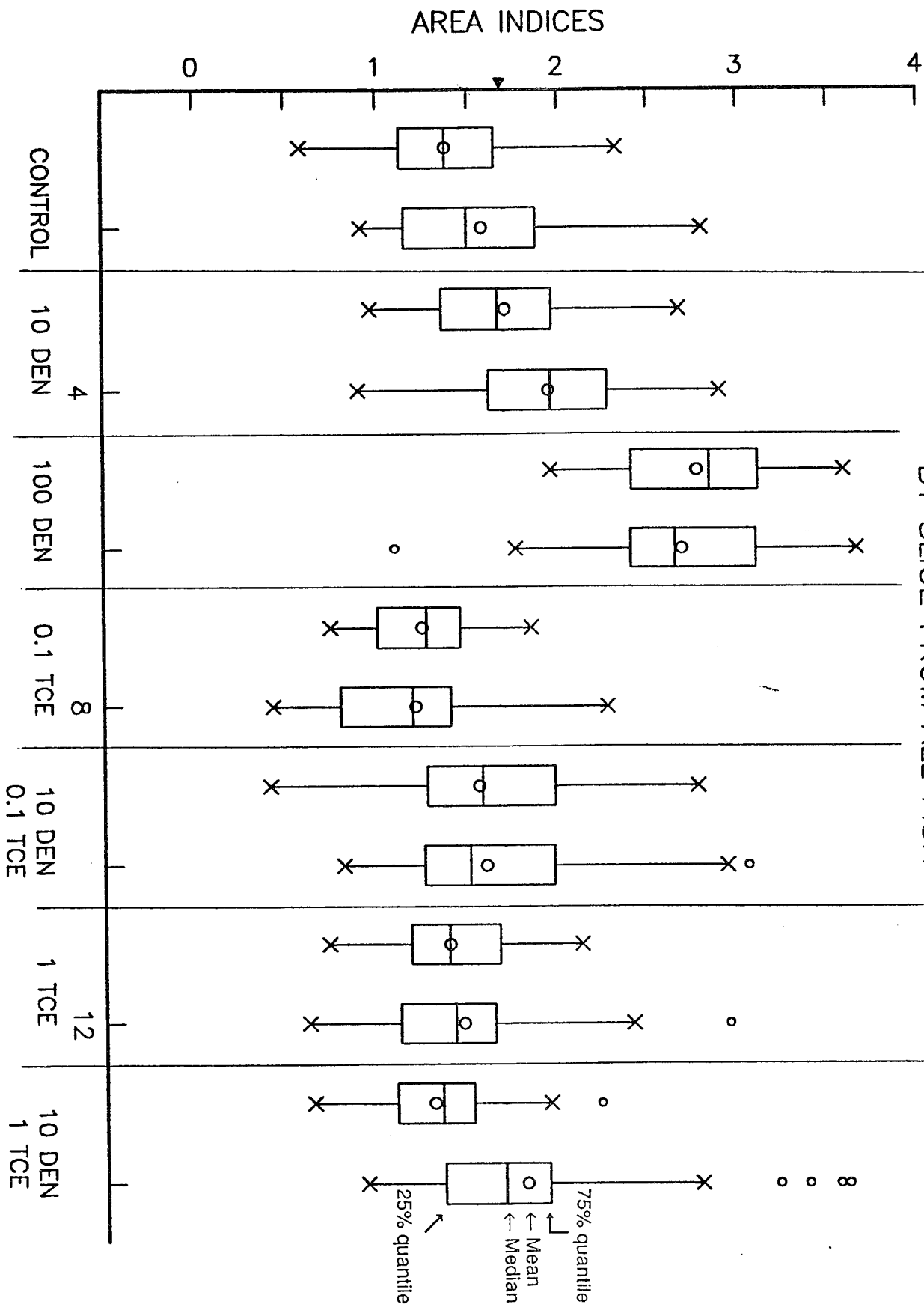


Figure 6b

SACRIFICE B: SQUARE ROOT OF AREA INDICES

MEAN OF TREATMENT MEANS - MEAN OF CONTROL MEANS
+/- 95 PERC TUKEY SIMUL CONF LIMITS FOR ALL DIFF

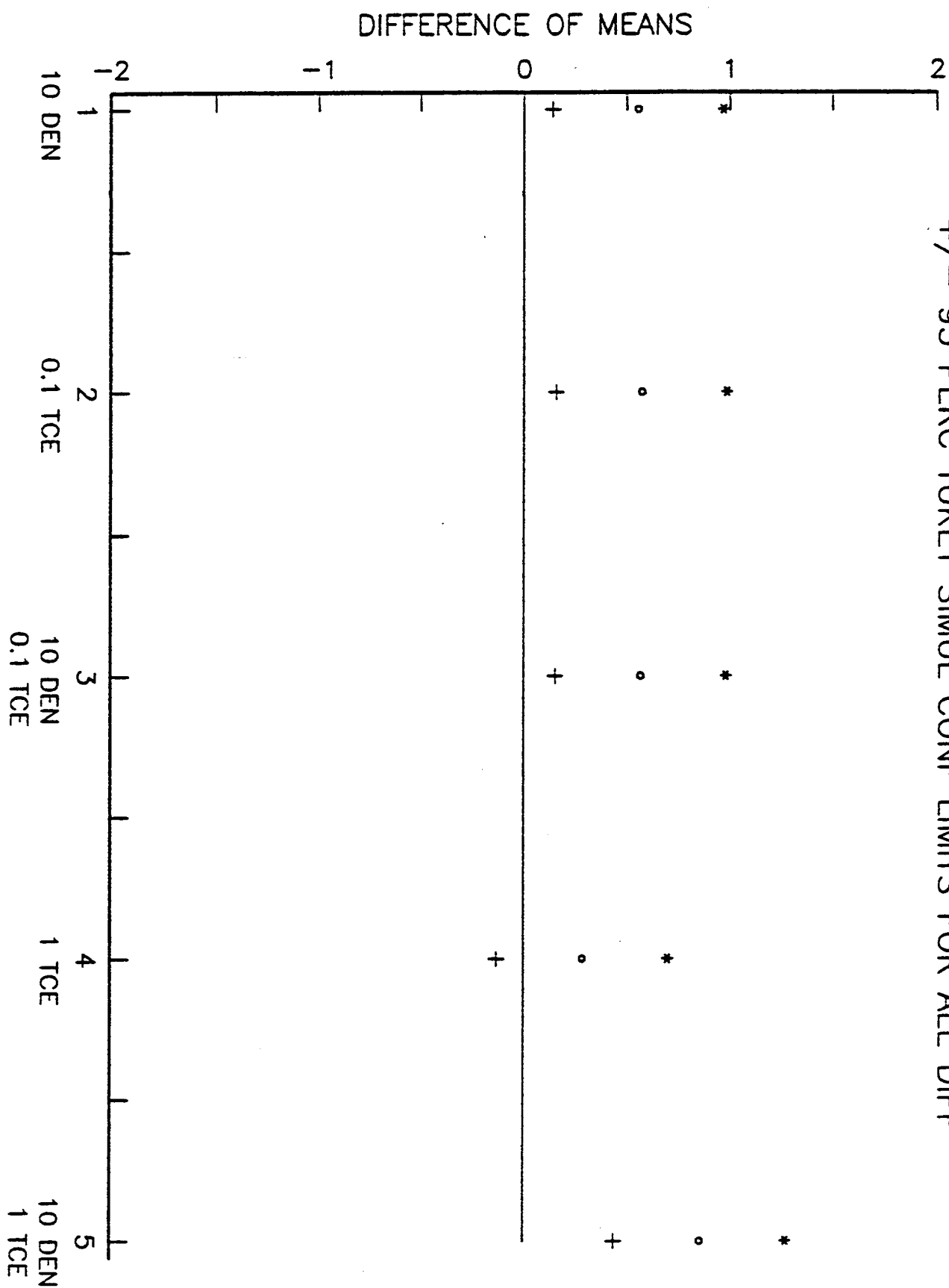


Figure 7

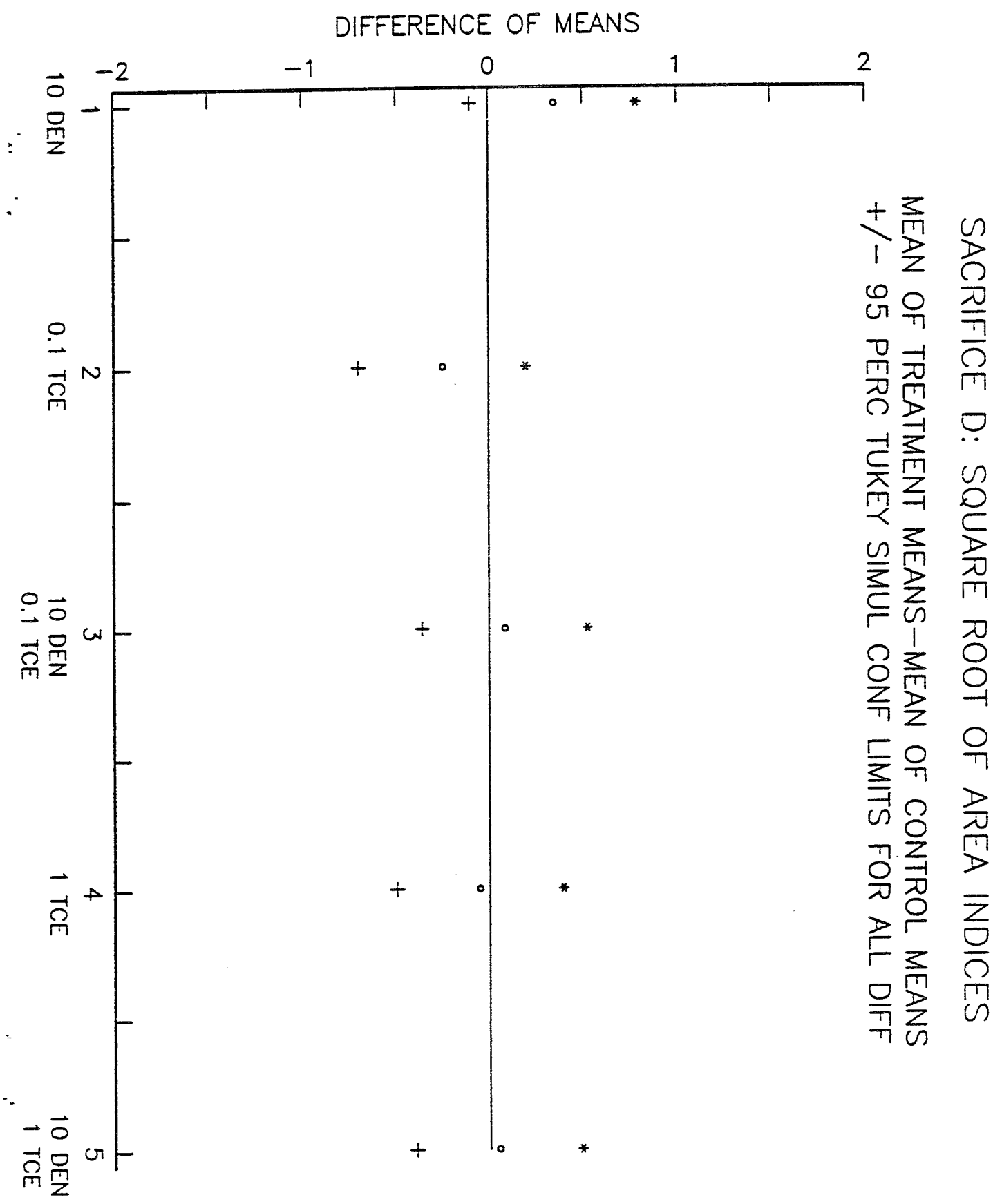


Figure 8

SACRIFICE B:MEAN(10 DEN)—MEAN(ODEN)

95 PERCENT STUDENTIZED RANGE SIMULTANEOUS CONFIDENCE INTERVALS
FOR ALL TREATMENT MEAN DIFFERENCES

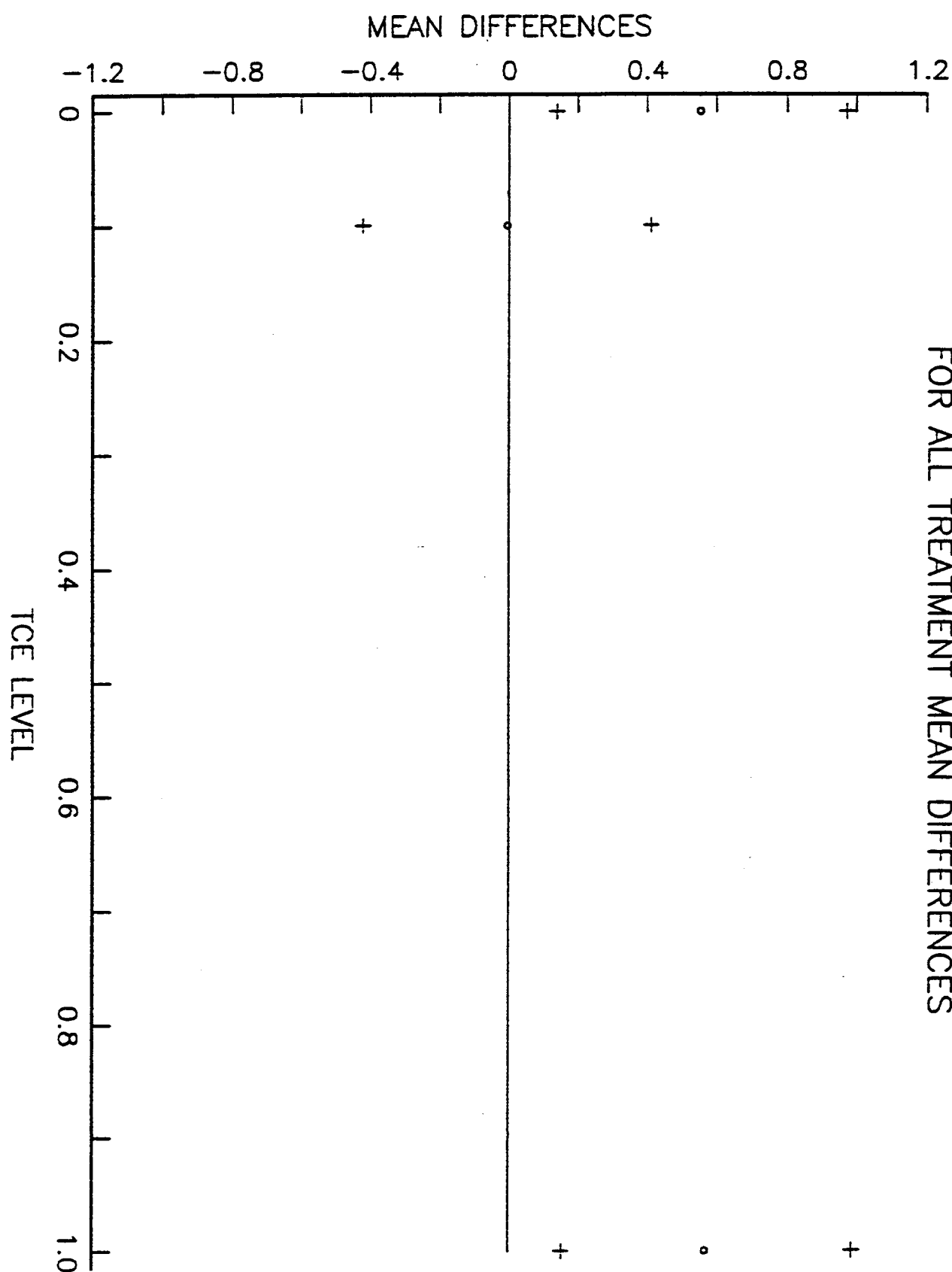


Figure 9

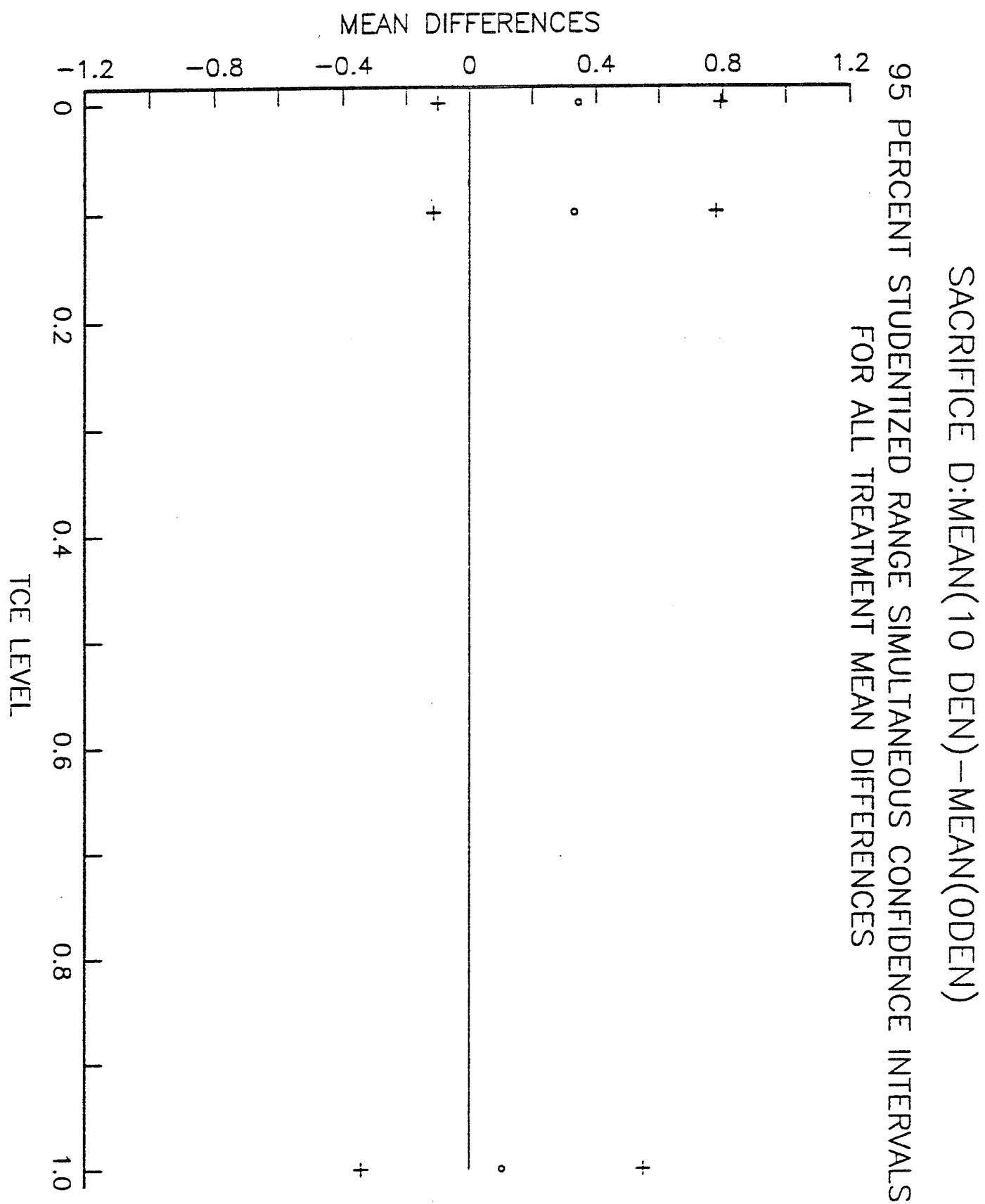


Figure 10

SQUARE ROOT OF AREA INDICES

MEAN OF MEANS BY TREATMENT FOR SACR D-SACR B
95 PERCENT CONF LIMITS OVER ALL DIFF

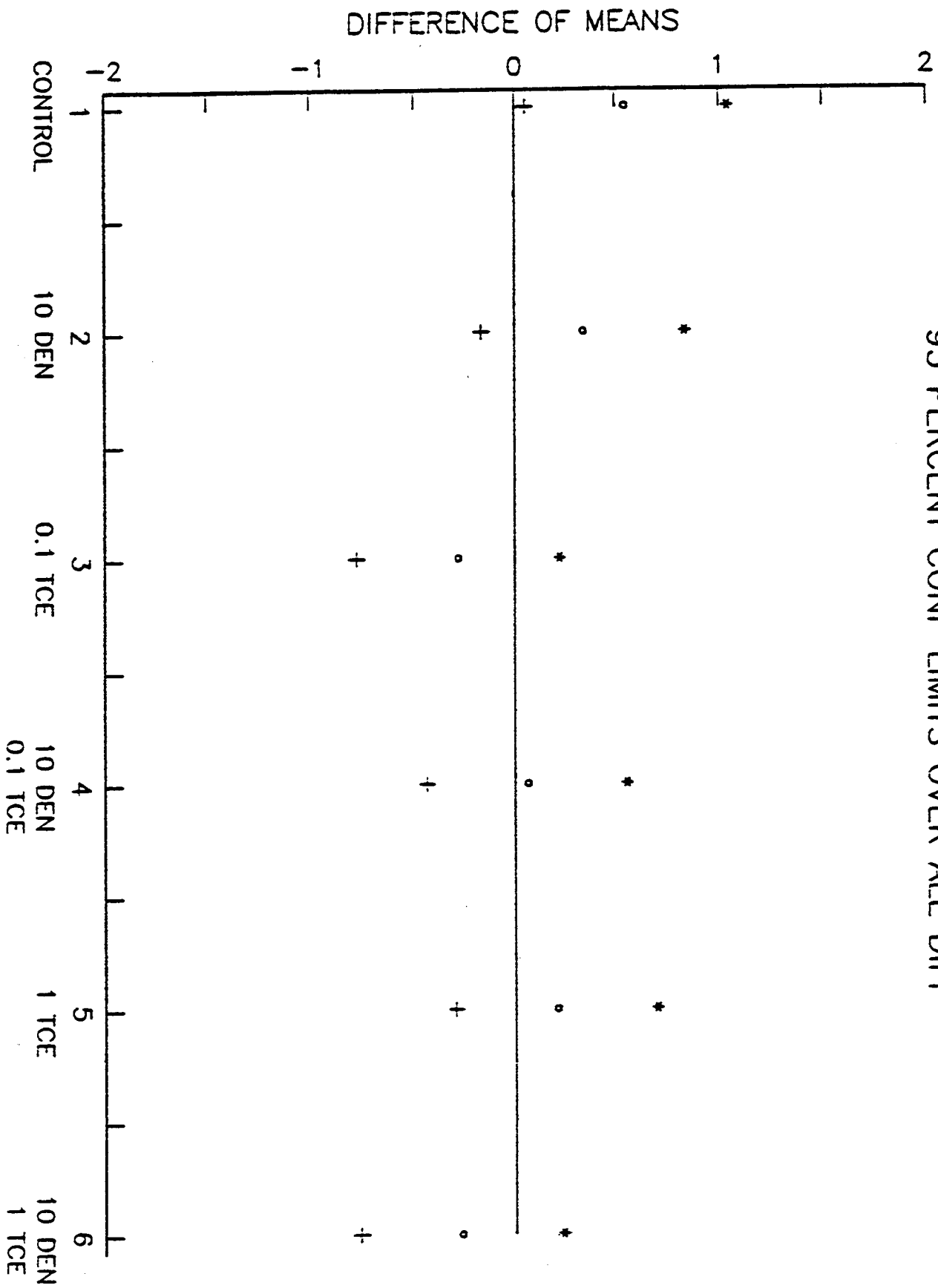


Figure 11

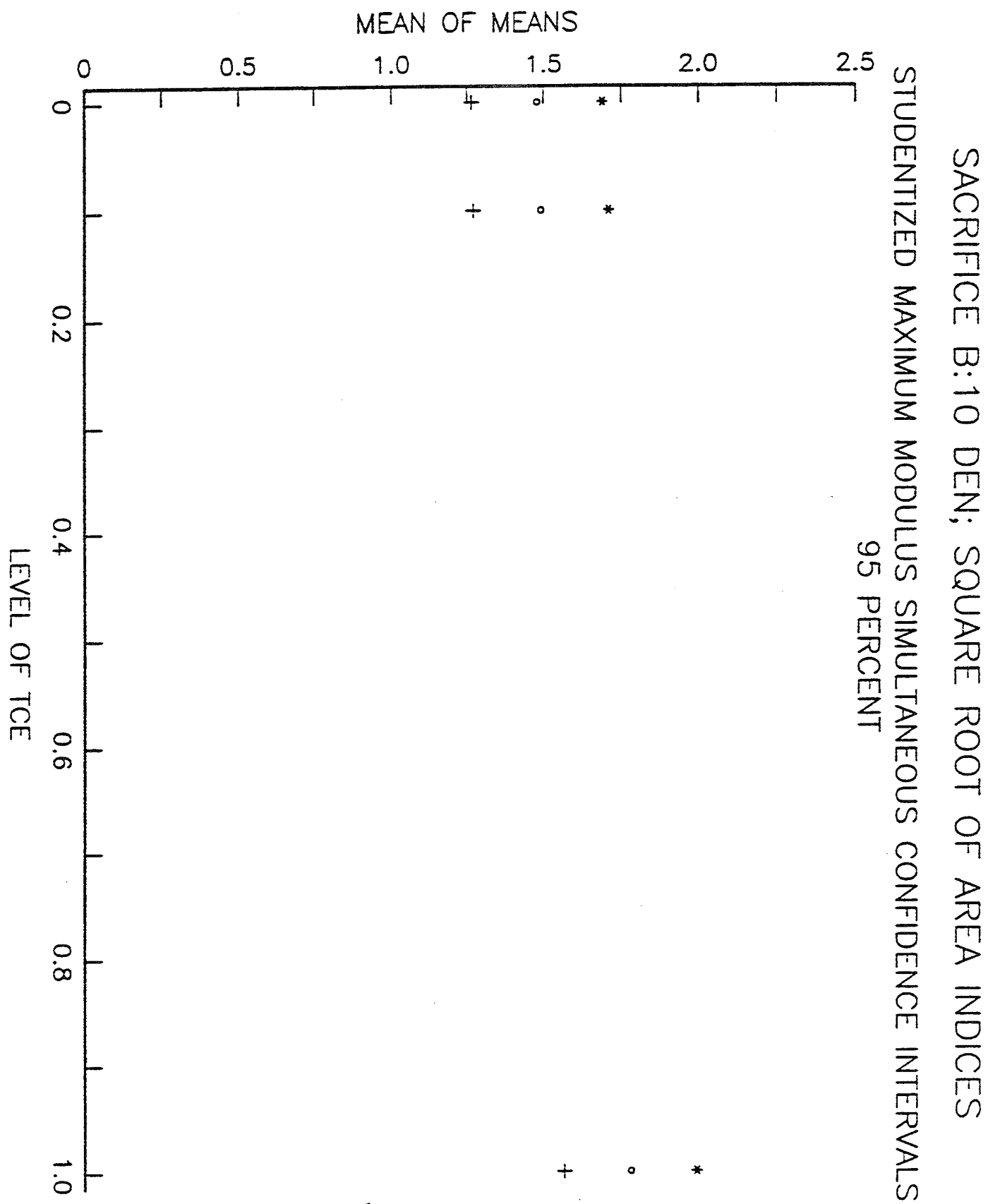


Figure 12
Modeling and Statistical Analysis of Bioassay Data: Medaka Cell Proliferation ...

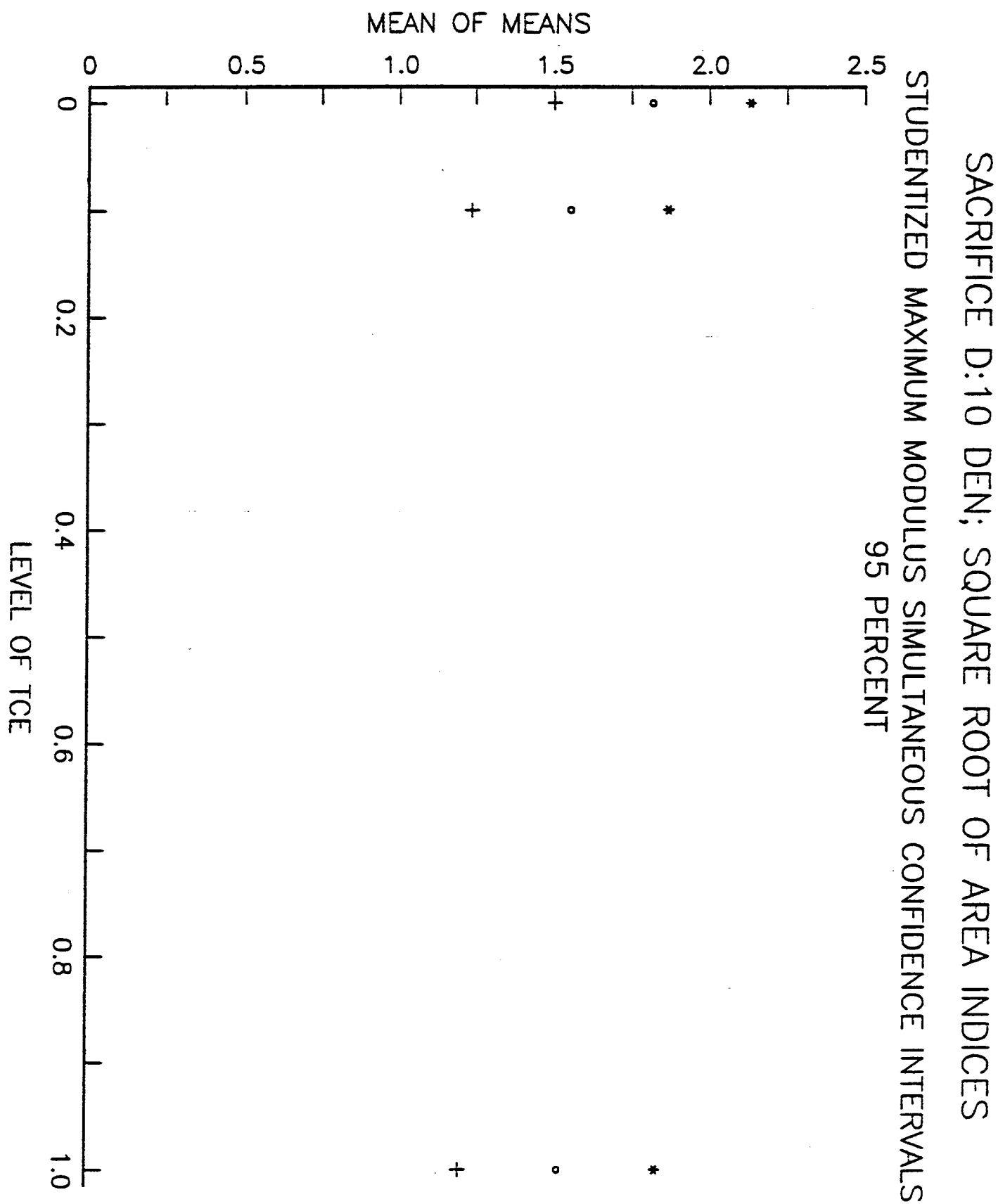


Figure 13

APPENDIX 5

An Analysis of Female Breast Tissue Data In Order to Predict Cancer

by

D. P. Gaver and P. A. Jacobs

**Operations Research Department
Naval Postgraduate School
Monterey, CA 93943**

1. Introduction

In October 1994, Dr. D. Malins sent us data dated 10/25/94 from a study of female breast tissues. The data are measurements from breast tissue samples from 30 female patients. Fifteen of the patients underwent reduction mammoplasty; tissues from these patients are considered to be normal. The other 15 patients had invasive ductal carcinoma; some of the samples from these patients are from breast tumors; other samples are from microscopically normal tissue from the cancer patients. The study used multiple breast tissue samples from some patients.

The data are measurements of hydroxymethyluracil (HMUra), fapyadenine (Fapy-A), 8-hydroxyadenine (8-OH-A), fapyguanine (Fapy-G), and 8-hydroxyguanine (8-OH-G) from the breast tissue samples.

In this study we restrict our attention to the samples from the women who underwent reduction mammoplasty (normal) and tissues from invasive ductal carcinoma tumors (cancer). There are 68 samples from women who underwent reduction mammoplasty and 10 samples from invasive ductal tumors for a total of 78 samples.

We are interested in the ability of the covariates Fapy-A, 8-OH-A, Fapy-G, and 8-OH-G to predict occurrence of cancerous/normal tissue. The data sample type was recoded to be equal to 0 for a sample from a reduction mammoplasty and 1 for a sample from an invasive ductal tumor. Logistic regression models (cf. Collett (1991)) are used to describe and predict data.

One way to evaluate the usefulness of a statistical model is to evaluate how well it describes the data used to estimate the model (goodness-of-fit). Another way is to evaluate how well the statistical model predicts new data that was not used to estimate its parameters. This latter process is called *cross validation*; it is a natural and well-accepted procedure for assessing the quality of a proposed prediction methodology. Mosteller and Tukey (1977) give a good discussion.

In this paper, simulation is used to evaluate logistic regression models with different numbers of covariates. In each simulation replication, the data are randomly allocated to one of two data sets. One data set is then used to estimate the parameters. The estimated model is then used to predict the probability that a data point from the other data set is from a cancerous tissue.

Summary

A summary of the results is as follows. Of the logistic regression models considered, the logistic regression model with the best goodness-of-fit is, not surprisingly, the one with the largest number of covariates: constant, log (Fapy-A), log (8-OH-A), log (Fapy-G), and log (8-OH-G). However, this model tends to be the weakest predictor. This model can be viewed as overfitting the data. There are two logistic regression models that are better predictors: regression Model I has these covariates: constant, log (Fapy-A), and log (8-OH-A); the other logistic regression model (II) has these covariates: constant and log (Fapy-A/8-OH-A). On the basis of the cross-validated

procedure described, regression Model II tends to predict the occurrence of normal samples somewhat better than Model I does. However, Model I predicts cancer samples somewhat better than Model II. What this means is that Model I tends to give more false positives than Model II, whereas Model II tends to give more false negatives than Model I. Model I describes the data it was fit to somewhat better than Model II; this is not surprising since it involves one more parameter than does Model II. All the logistic regression models considered had more difficulty in predicting the cancer samples than they did with normal samples: on the average about 1 out of 5 cancer samples was incorrectly predicted.

Section 2 describes the logistic regression model. Section 3 describes a simulation experiment which results in classifications and Section 4 reports results from that simulation. Sections 5 and 6 describe additional simulation experiments and present their results.

2. The Logistic Regression Model

Suppose we have responses which can either be 0 (from normal tissue) or 1 (from cancer tumor) and let p_i be the probability that the i^{th} response is cancerous. The *logistic regression model* for the dependence of p_i on the values of k exploratory variables $x_{i1}, x_{i2}, \dots, x_{ik}$ associated with the i^{th} response is

$$\begin{aligned}\text{logit}(p_i) &= \log(p_i/(1-p_i)) \\ &= \beta_0 + x_{i1}\beta_1 + x_{i2}\beta_2 + \dots + x_{ik}\beta_k.\end{aligned}\tag{2.1}$$

After rearrangement

$$p_i = \frac{\exp\{\beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \dots + \beta_k x_{ik}\}}{1 + \exp\{\beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \dots + \beta_k x_{ik}\}}.\tag{2.2}$$

In what follows we use the procedure in the standard statistical package S-PLUS to estimate the parameters of the logistic regression models.

Note that the data analysis conducted to date has been confined to use of the logistic model exclusively, and to expression of the covariates or explanatory variables in terms of the logarithms of their concentrations. Other options for the link function (connection between response probability and covariates, here the logistic) and covariate representation could profitably be investigated.

It is strongly recommended that development of a truly mechanistic model that represents biological process be conducted, and the latter used for prediction. Work in this direction has been initiated with Dr. J. Burkhardt of NIEHS, and should extend well to the present problem.

3. A Simulation Experiment Resulting in Classifications

Each simulation experiment consists of 100 replications. In what follows an observation consists of type of sample (0 = normal, 1 = cancerous) and the corresponding covariates.

Each replication consists of the following operations. Each observation or data point is randomly allocated to the data set used to fit the statistical model (FD) or to the data set which the fitted model is used to predict (PD). If the data set used to fit the model contains no cancer samples the randomization is done again. Thus, on the average, 1/2 of the observations are used to fit the statistical model and 1/2 of the observations are reserved to assess the predictive ability of the fitted model. Some FD data sets contain as few as 1 cancer sample, so the prediction assessments may be conservative. Other sampling procedures are described in Section 5.

One or more logistic regression models are estimated using the data set FD. The parameter estimates are then used in (2.2) to evaluate the predicted

probability of the sample being cancerous for each observation in the data set PD. In order to make a Yes–No statement a probability *cut point* must be chosen; this is actually a parameter. For this study we choose $p = 0.5$: if the predicted probability is less than or equal to 0.5, a classification of 0 is assigned to the observation; if the predicted probability is greater than 0.5, a classification of 1 is assigned to the observation.

In order to assess classification accuracy the absolute difference between the classification and the sample type (recoded to be 0 for normal and 1 for cancerous) is computed for each observation in PD. The mean of the absolute difference is computed for the normal observations. The mean of the absolute differences is computed for the cancer observations. Note that perfect prediction classification would result in a mean of 0. If all the predictions are wrong, then the mean will be 1. Thus, the mean measures the fraction of the predictions that are in error.

The goodness of fit is also evaluated. The procedure is as follows. The fitted probability of being cancerous is evaluated for each data point in FD. If the fitted probability is less than or equal to 0.5, a classification of 0 is assigned to the observation; if the fitted probability is greater than 0.5, then a classification of 1 is assigned to the observation. The absolute difference between the classification and the sample type (recoded to be 0 for normal and 1 for cancerous) is computed for each observation in FD. The mean of the absolute difference is computed for the normal observations. The mean of the absolute differences is computed for the cancer observations.

Note that a perfect fit classification would result in a mean of 0. If all the fit classifications were wrong, then the mean would be 1. Thus, the mean represents the fraction of fit classifications that are in error.

4. The Classification Simulation Results

The simulation results for the following logistic regression models are reported. All logistic regressions have a constant term.

<u>Model</u>	<u>Covariates</u>
I:	$\log(\text{Fapy-A}), \log(8\text{-OH-A})$
II:	$\log(\text{Fapy-A}/8\text{-OH-A})$
III:	$\log(\text{Fapy-A}/8\text{-OH-A}), \log((\text{Fapy-A}) \times (8\text{-OH-A}))$
IV:	$\log(\text{Fapy-A}), \log(8\text{-OH-A}), \log(\text{Fapy-G}), \log(8\text{-OH-G})$
V:	$\log(\text{Fapy-A}/8\text{-OH-A}), \log(\text{Fapy-G}/8\text{-OH-G})$

All of these models were estimated using the same sets of data and used to predict the same sets of data. Note that Model I and Model III are equivalent but the parameterization of Model III may be more biologically meaningful.

Table 1 reports the mean of the mean fit-classification errors. Model IV with the greatest number of covariates (not surprisingly) gives the best fit.

Table 2 reports the mean of the mean predicted-classification errors. Note that the 5 fitted logistic regression models are all predicting the same sets of data and were all estimated using the same sets of data. Thus, the means are comparable. The best fitting Model IV predicts less well than Models I, II and III. Model II appears to categorize the normal samples the best but does not categorize the cancer samples as well as Models I (and III). Thus, Model I (and III) tends to give more false positives than Model II. However, Model II tends to give more false negatives than Model I (and III). Since there are on the average 5 cancer observations to be predicted, the results suggest that on the average one or two of the cancer observations are misclassified as normal for all of the models. Since there are on the average 34 normal observations to be predicted, the results

suggest that Models I – III misclassify about one normal observation as cancerous ($1/34 = 0.029$).

5. A Balanced Simulation Experiment

Each simulation experiment consists of 100 replications. Each replication consists of the following operations.

34 of the 68 normal observations, and 5 of the 10 cancer observations, are chosen randomly without replacement to be in the data set FD. The other normal and cancer observations are put in data set PD.

The parameters of the logistic regression models are estimated using the data in FD. The fitted probability of the observation being cancerous is computed using (2.2) and the parameter estimates for each observation in FD. The mean absolute difference between the fitted probability and the sample type (0 = normal, 1 = cancerous) is computed for all normal observations (respectively all cancer observations).

The estimated parameters are then used in (2.2) to evaluate the predicted probability of the sample being cancerous for each observation in the data set PD. The mean absolute difference between the predicted probability and the sample type is computed for all normal observations (respectively all cancer observations).

Once again perfect fit (respectively prediction) would result in a mean of 0. A poor fit (respectively prediction) will have a mean closer to 1.

6. Results of Simulation Experiments

Tables 3 and 4 report the results of simulation experiments comparing the logistic regression models with the following covariates.

<u>Model</u>	<u>Covariates</u>
I:	Constant, log (Fapy-A), log (8-OH-A)
II:	Constant, log (Fapy-A/8-OH-A)
III:	Constant, log (Fapy-A/8-OH-A), log ((Fapy-A) \times (8-OH-A))
IV:	Constant, log (Fapy-A), log (8-OH-A), log (Fapy-G), log (8-OH-G)
V:	Constant, log (Fapy-A/8-OH-A), log (Fapy-G/8-OH-G)

Tables 5 and 6 report results of simulation experiments comparing the logistic regression models with the following covariates.

<u>Model</u>	<u>Covariates</u>
Ia:	Constant, log (Fapy-A)
IIa:	Constant, log (8-OH-A)
IIIa:	Constant, log (Fapy-A/8-OH-A)
IVa:	Constant, log (Fapy-A), log (8-OH-A)
Va:	Constant, log (Fapy-A/8-OH-A), log (Fapy-G/8-OH-G)

Once again Model IV offers the best fit. However, it tends to over-predict the presence of cancer in normal tissue and under-predict the presence of cancer in cancer tissue. Model I (IVa) with covariates: constant, log (Fapy-A) and log (8-OH-A) (not surprisingly) has smaller mean absolute fitted error than Model II (IIIa) with covariates: constant and log (Fapy-A/8-OH-A). In terms of prediction, the mean of the mean prediction errors for normal samples for Model I (IVa) is greater than or equal to that for Model II (IIIa). However, the mean of the mean prediction errors for the cancer samples for Model II (IIIa) is greater than that for Model I (IVa).

7. Conclusions

In this paper we evaluated the usefulness of logistic regression models in two ways, goodness-of-fit and ability to predict. The best fitting logistic regression model did not predict new observations as well as some of the other models. Two better predicting logistic regression models are

<u>Model</u>	<u>Covariates</u>
I:	Constant, log (Fapy-A), log (8-OH-A)
II:	Constant, log (Fapy-A/8-OH-A)

Both of these models, used as described above, tend to give false negatives in about 1 out of 5 cancer samples. Both tend to give false positives in about 1 out of 34 normal samples.

REFERENCES

- Statistical Sciences, Inc., *S-PLUS for Windows*, Version 3.1, Statistical Sciences, Inc. Seattle, WA, 1993.
- D. Collett, *Modeling Binary Data*, Chapman and Hall, New York, NY, 1991.
- F. Mosteller and J. W. Tukey, *Data Analysis and Regression*, Addison-Wesley, Reading, MA, 1977.

Table 1
Fitted Values
Mean of Mean Absolute Fitted Classification Error

Model	<i>Normal Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)	<i>Cancer Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)
I	0.011 (0.015)	0.159 (0.145)
II	0.016 (0.016)	0.186 (0.143)
III	0.011 (0.015)	0.159 (0.145)
IV	0.000 (0.000)	0.000 (0.000)
V	0.016 (0.019)	0.165 (0.147)

Table 2
Predictions
Mean of Mean Absolute Prediction Classification Error

Model	<i>Normal Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)	<i>Cancer Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)
I	0.027 (0.031)	0.231 (0.226)
II	0.025 (0.026)	0.243 (0.228)
III	0.027 (0.031)	0.231 (0.226)
IV	0.050 (0.045)	0.265 (0.241)
V	0.030 (0.034)	0.267 (0.249)

Model **Covariates**

- I: Constant, log (Fapy-A), log (8-OH-A)
- II: Constant, log (Fapy-A/8-OH-A)
- III: Constant, log (Fapy-A/8-OH-A), log ((Fapy-A) \times (8-OH-A))
- IV: Constant, log (Fapy-A), log (8-OH-A), log (Fapy-G), log (8-OH-G)
- V: Constant, log (Fapy-A/8-OH-A), log (Fapy-G/8-OH-G)

Table 3
Fitted Values
Mean of Mean Absolute Fitted Error

Model	<i>Normal Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)	<i>Cancer Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)
I	0.0252 (0.0203)	0.171 (0.138)
II	0.0288 (0.0196)	0.196 (0.134)
III	0.0252 (0.0203)	0.171 (0.138)
IV	0.000 (0.000)	0.000 (0.000)
V	0.0271 (0.0205)	0.184 (0.140)

Table 4
Predictions
Mean of Mean Absolute Prediction Error

Model	<i>Normal Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)	<i>Cancer Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)
I	0.0548 (0.0448)	0.202 (0.138)
II	0.0486 (0.0329)	0.234 (0.164)
III	0.0548 (0.0479)	0.202 (0.138)
IV	0.0679 (0.0519)	0.209 (0.183)
V	0.0566 (0.0430)	0.235 (0.180)

Model Covariates

- I: Constant, log (Fapy-A), log (8-OH-A)
- II: Constant, log (Fapy-A/8-OH-A)
- III: Constant, log (Fapy-A/8-OH-A), log ((Fapy-A) × (8-OH-A))
- IV: Constant, log (Fapy-A), log (8-OH-A), log (Fapy-G), log (8-OH-G)
- V: Constant, log (Fapy-A/8-OH-A), log (Fapy-G/8-OH-G)

Table 5
Fitted Values
Mean of Mean Absolute Fitted Error

Model	<i>Normal Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)	<i>Cancer Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)
Ia	0.057 (0.021)	0.391 (0.145)
IIa	0.118 (0.009)	0.804 (0.063)
IIIa	0.030 (0.019)	0.202 (0.128)
IVa	0.026 (0.020)	0.176 (0.137)
Va	0.028 (0.020)	0.190 (0.137)

Table 6
Predictions
Mean of Mean Absolute Prediction Error

Model	<i>Normal Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)	<i>Cancer Sample</i> Mean of Replic. Means ($\sqrt{\text{Var. of Replic. Means}}$)
Ia	0.059 (0.030)	0.442 (0.117)
IIa	0.127 (0.023)	0.841 (0.042)
IIIa	0.04001 (0.027)	0.256 (0.163)
IVa	0.04002 (0.028)	0.225 (0.125)
Va	0.045 (0.033)	0.273 (0.192)

Model **Covariates**

- Ia: Constant, log (Fapy-A)
- IIa: Constant, log (8-OH-A)
- IIIa: Constant, log (Fapy-A/8-OH-A)
- IVa: Constant, log (Fapy-A), log (8-OH-A)
- Va: Constant, log (Fapy-A/8-OH-A), log (Fapy-G/8-OH-G)

APPENDIX 6

Remarks Resulting From a Preliminary Examination of Data Sent by Dr. L. Twerdok of GEO-CENTERS, INC. at U.S. Army BRDL in October 1994

by
P. A. Jacobs and D. P. Gaver
Department of Operations Research
Naval Postgraduate School
Monterey, CA 93943

1. Introduction

The data consist of measurements made on medaka that were sacrificed at different times.

The information recorded for each fish include: the date of the experiment (which is called the sacrifice data here); the age (in months); the length in millimeters; the weight in milligrams; percent hematocrit; percent leukocrit; and the hatch date. The minimum recorded value of leukocrit is 0.01; this value is a code for "below the limit of detection".

There are missing values which are coded by the value 100. There are 2 suspect weights; one of 1079 mg for one of the fish sacrificed on 3/16/92 and one equal to 33 mg for one of the fish sacrificed on 7/25/94.

2. Graphical Displays

Only the data without any missing values are considered for this preliminary examination of the data. Since all of the remaining sacrifice dates are in 1994, in Figures 1–4 the sacrifice dates are coded as follows: 7/25/94 is coded as 725; 10/05/94 is coded as 1005, etc.

2.1 Boxplots by Hatch Date

Figure 1 displays boxplots of log (leukocrit) as a function of the sacrifice date; the leukocrit values of 0.01 are not considered. There is one figure for each hatch

date. A remarkable feature is the apparent decline in the log (leukocrit value) for later sacrifice dates for the 1/27/94 hatch date.

Figure 2 displays boxplots of log (hematocrit) as a function of sacrifice date. There is one figure for each hatch date. There does not appear to be as much systematic variability of log (hematocrit) between sacrifice times as there is for log (leukocrit); however note that the scales are different.

Figure 3 (respectively Figure 4) displays boxplots of length (respectively weight) by sacrifice date. There is one figure for each hatch date. There are sometimes apparent declines in length and weight for later sacrifice times.

Figures 5–8 display boxplots of measurements by *age* of the sacrificed fish for each hatch date. There is one figure for each hatch date. Figure 5 displays boxplots for log (leukocrit) without the 0.01 values. There is an apparent decrease in log (leukocrit) numerical value with age. Figure 6 displays boxplots of log (hematocrit). Figure 7 (respectively Figure 8) displays boxplots of length (respectively weight) by age for each hatch date. Once again there is sometimes the (surprising?) apparent decrease in length and weight numerical values with age.

Summary. There is the suggestion that the measurements are associated with sacrifice date beyond what is expected to be age dependence. The leukocrit measurement seems to be particularly variable across sacrifices. This may suggest the presence of a “sacrifice effect” that could affect the problem of treatment comparisons across sacrifices. Note that no formal statistical analysis has yet been conducted to quantify the strength of the effect.

2.2 Associations By Age

Figure 9 is a display of scatterplots of log (weight) versus log (length) by age of fish at time of sacrifice; there is one plot for each fish age. Not surprisingly, there appears to be an association.

Figure 10 is a display of scatterplots of log (hematocrit) versus log (leukocrit) by age of fish at time of sacrifice; the leukocrit values of 0.01 are omitted. There is little consistent association across ages, although there is a hint that log (leukocrit) and log (hematocrit) increase together at ages 3 and 5, are negatively or inversely related at ages 6–8, and become essentially unrelated after age 9.

Figure 11 is a display of boxplots of log (hematocrit) by age. An analysis of variance does not reject the null hypothesis that the means are all equal; ($p = 0.89$).

Figure 12 is a display of log (leukocrit) by age along with an estimated least squares straight line; those values of leukocrit = 0.01 have been omitted from consideration. The estimated least squares linear line is

$$\begin{array}{rcl} \log \text{ leukocrit} & = & 0.89 - 0.17 (\text{age}) \\ (\text{s.e.}) & & (0.16) \quad (0.02) \end{array} \quad (2.1)$$

The estimated equation is displayed along with estimated standard errors for the parameter values in parentheses. Since the standard error of the slope is less than 2 times the absolute value of the estimate of the slope, there is an apparent decrease in log (leukocrit) with age. However, recall that the level of leukocrit also appears to be associated with sacrifice date. An age grouping may contain several sacrifice dates. If there is a “sacrifice effect” this would tend to dilute the strength of the relationship explored.

Figure 13 displays boxplots of the residuals from the least squares regression by sacrifice data. An analysis of variance rejects the null hypothesis that the residual means are equal for the different sacrifice dates. This is in agreement with the low R^2 discovered in the regression of Figure 12, and tends to support the hypothesis that there may be a “sacrifice effect”.

The following least squares regression model was estimated (omitting the leukocrit values of 0.01)

$$\log(\text{leukocrit}) = b_0 + b_1(\text{age}) + b_2(\text{length})$$

The $R^2 = 0.31$ with residual standard error = 0.60. The parameter estimates (with estimated standard errors) are

$$\begin{array}{ccc} b_0 = 2.7 & b_1 = -0.14 & b_2 = -0.07 \\ (0.44) & (0.02) & (0.02) \end{array}$$

An analysis of variance of the residuals from the regression versus sacrifice date rejects the null hypothesis of equal means across sacrifice date ($F = 7.3$ with between-df = 15 and within-df = 198, $p = 2 \times 10^{-12}$). Again there seems to be a source of variability associated with sacrifices.

Figure 14 displays boxplots of the residuals of the regression versus sacrifice date. Note the apparent decline in the residuals for the last cluster of experiments conducted from 10/3/94 through 10/18/94.

3. Preliminary Conclusions

There does not appear to be an association between age and log (hematocrit) level. A least squares linear regression (2.1) suggests that there may be an association between age and leukocrit. However, analysis of variance of the residuals from the least squares regression suggests an association between leukocrit and the sacrifice date. This association may be due to the procedure used to measure leukocrit; it may also be due to differences in water quality and other physical factors in the experiment and the health of the animals. If there are sacrifice effects they will tend to dilute the strength of associations between other measured variables such as age, hematocrit, leukocrit, length, weight, etc. Since we have been unable to discover a biological reason for a relationship between leukocrit value and age in adult animals, we suspect that age is a surrogate for some other effect.

EACH FIGURE IS FOR 1 HATCH DATE; 1 BOX PLOT FOR EACH SACR TIME

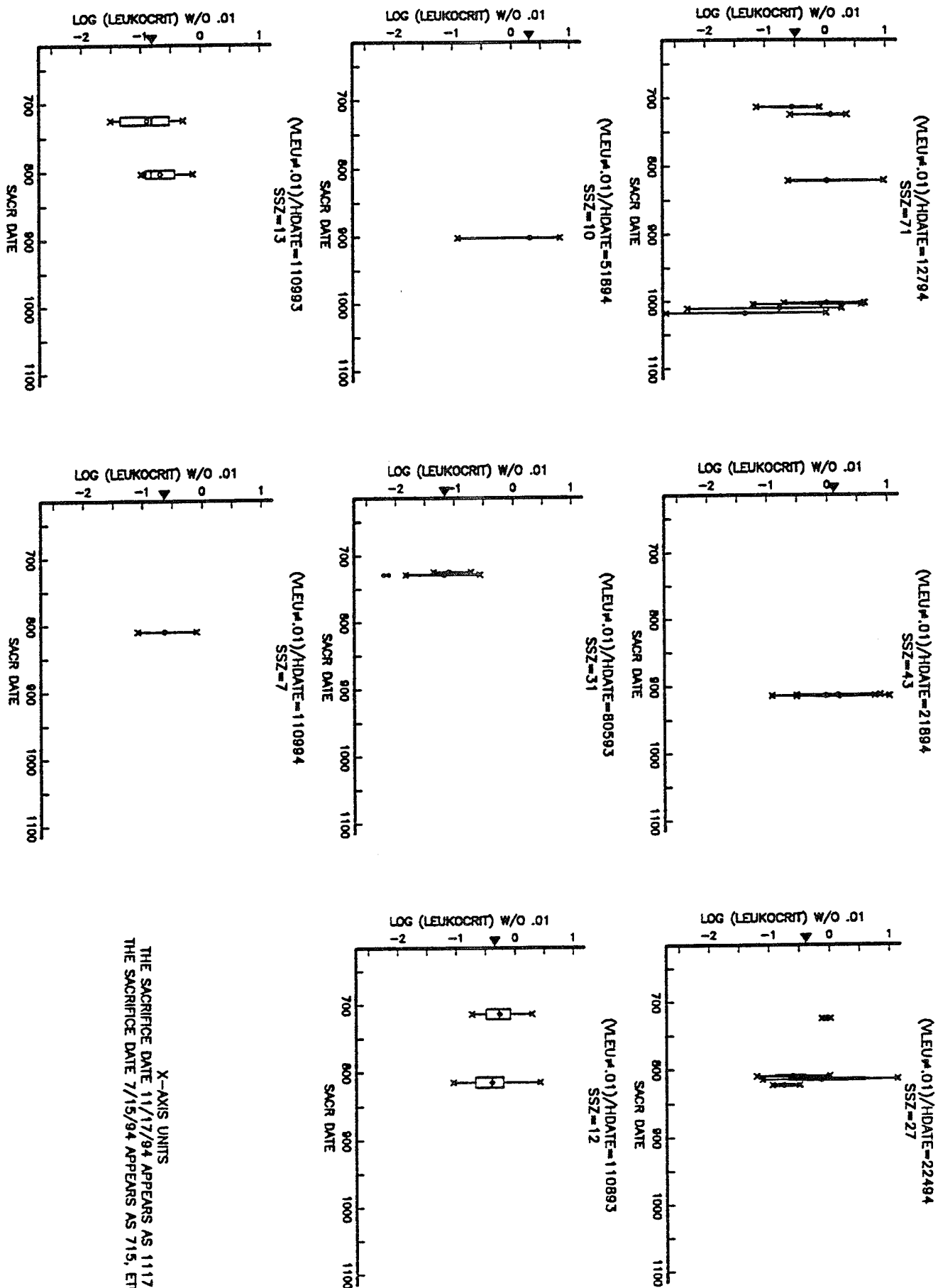


Figure 1

EACH FIGURE IS FOR 1 HATCH DATE; 1 BOX PLOT FOR EACH SACR TIME

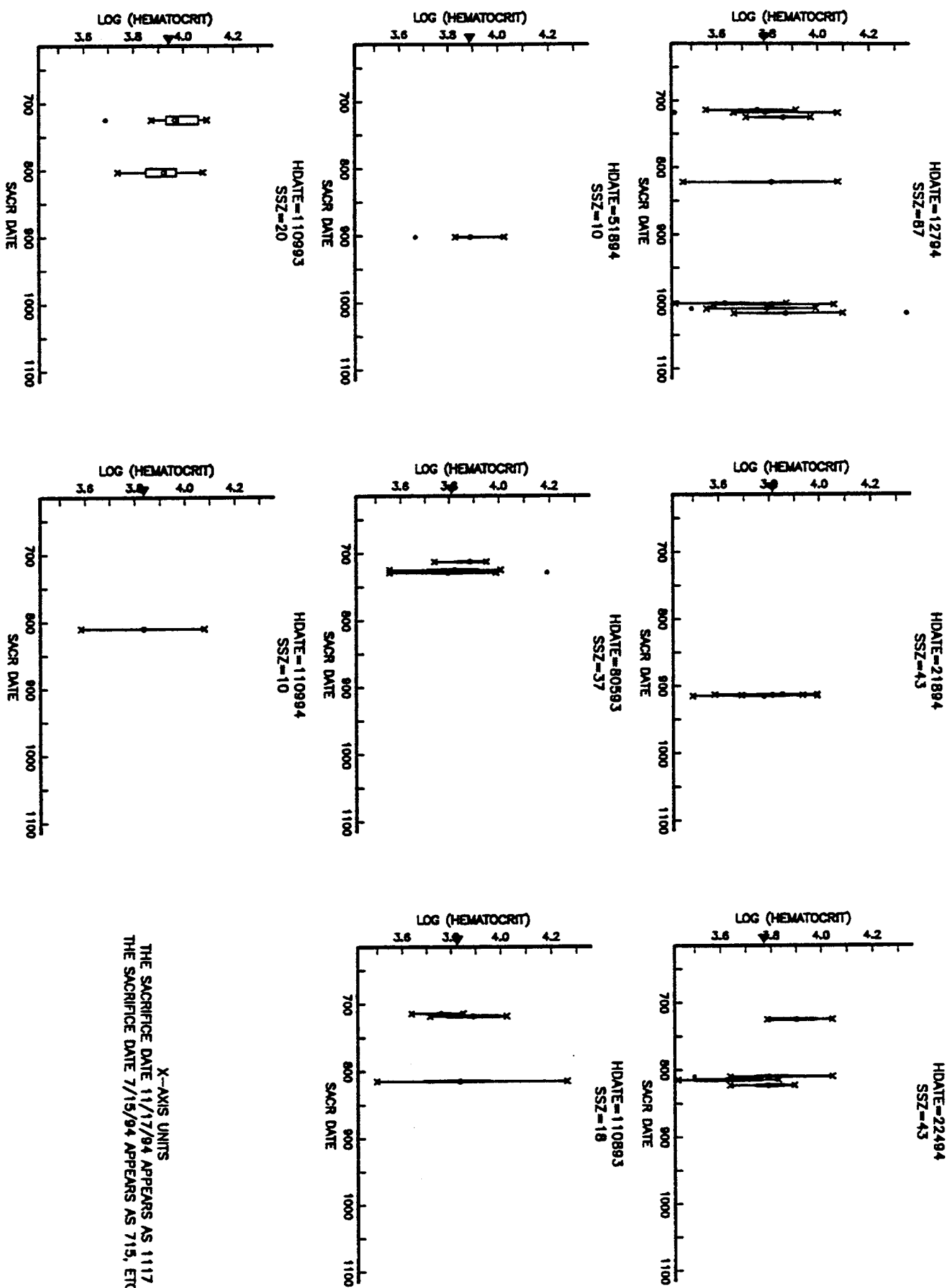


Figure 2

EACH FIGURE IS FOR 1 HATCH DATE; 1 BOX PLOT FOR EACH SACR TIME

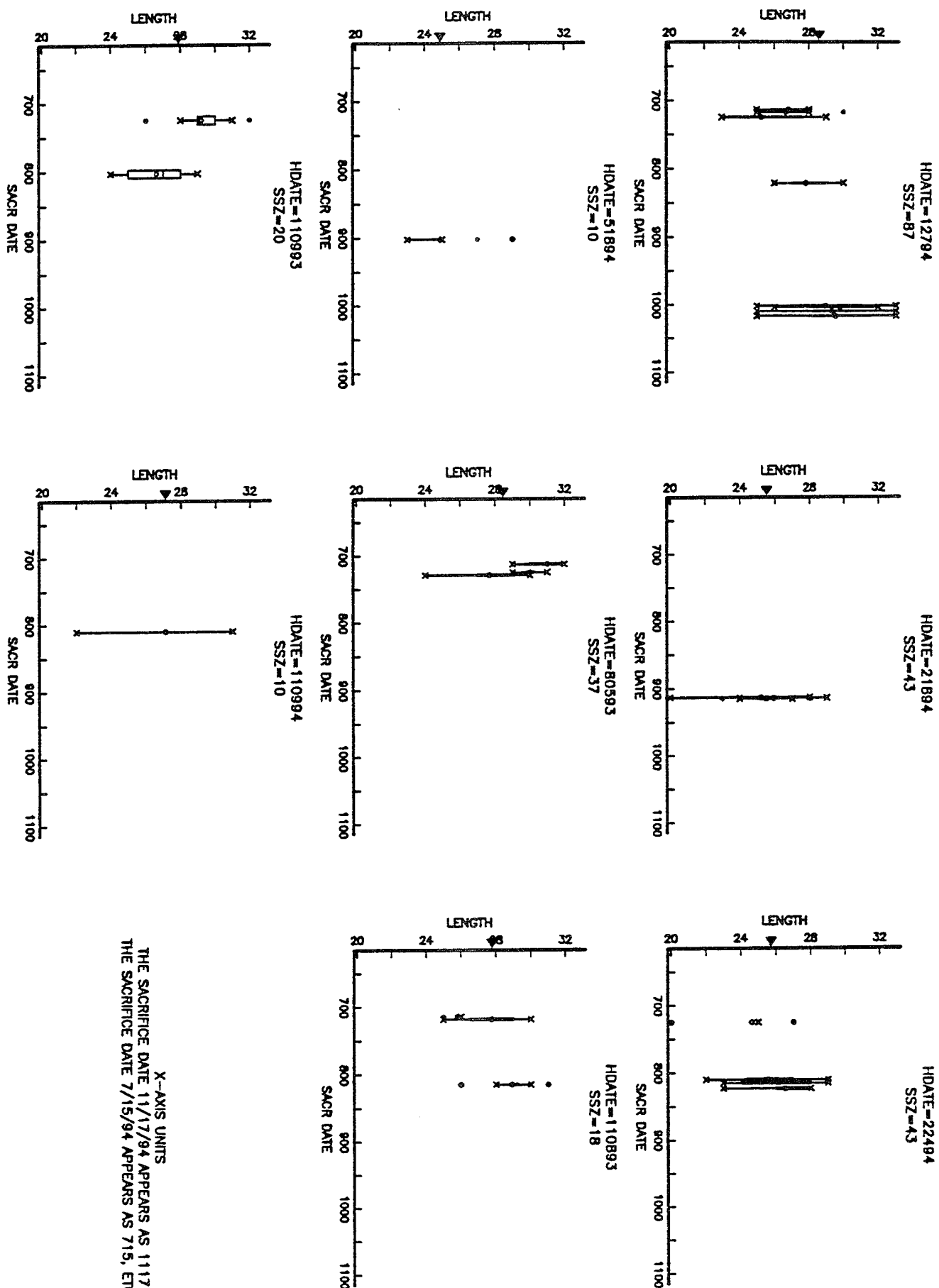


Figure 3

EACH FIGURE IS FOR 1 HATCH DATE; 1 BOX PLOT FOR EACH SACR TIME

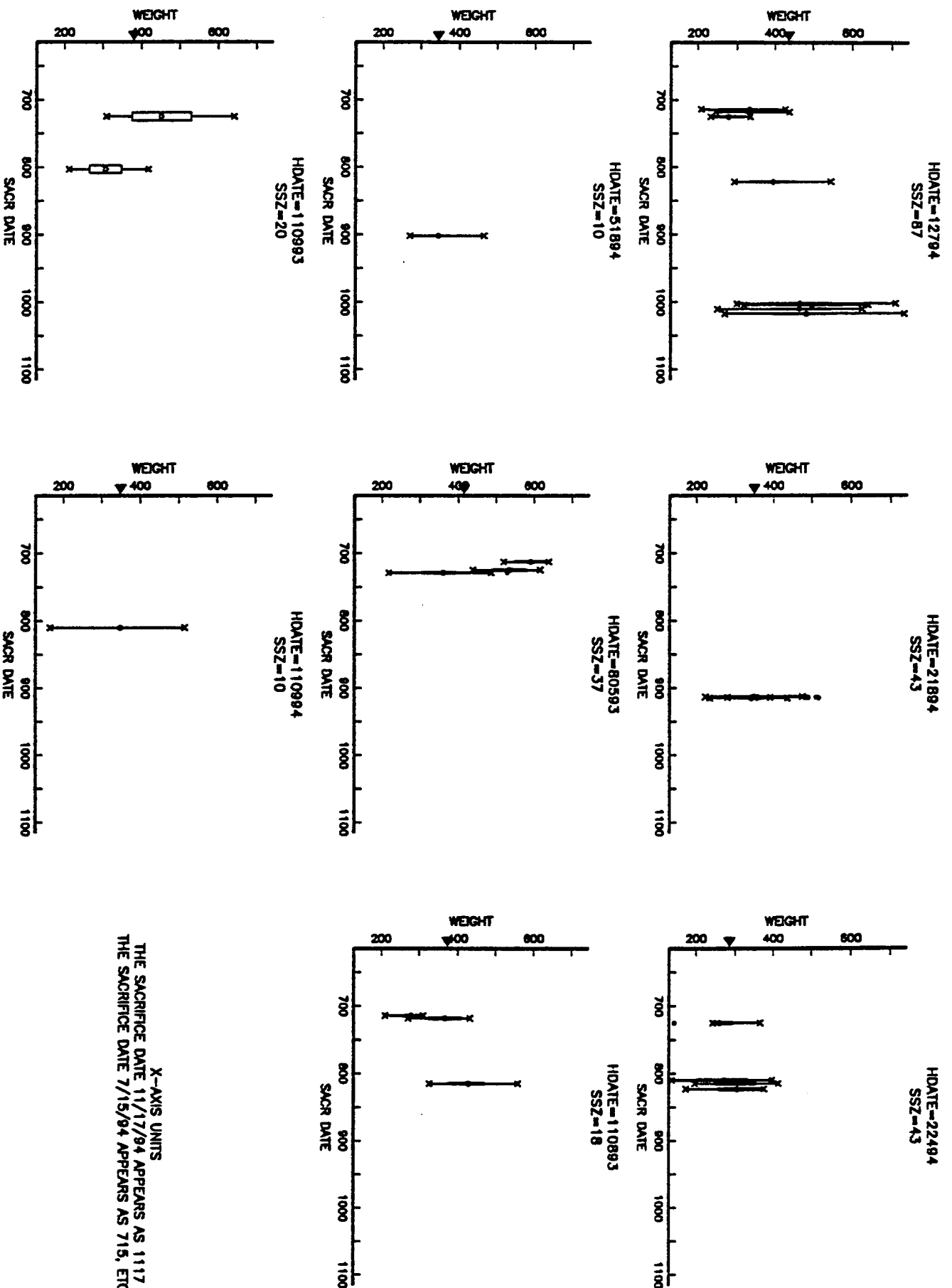


Figure 4

1 FIGURE BY HATCH DATE;1 BOX PLOT FOR EACH AGE

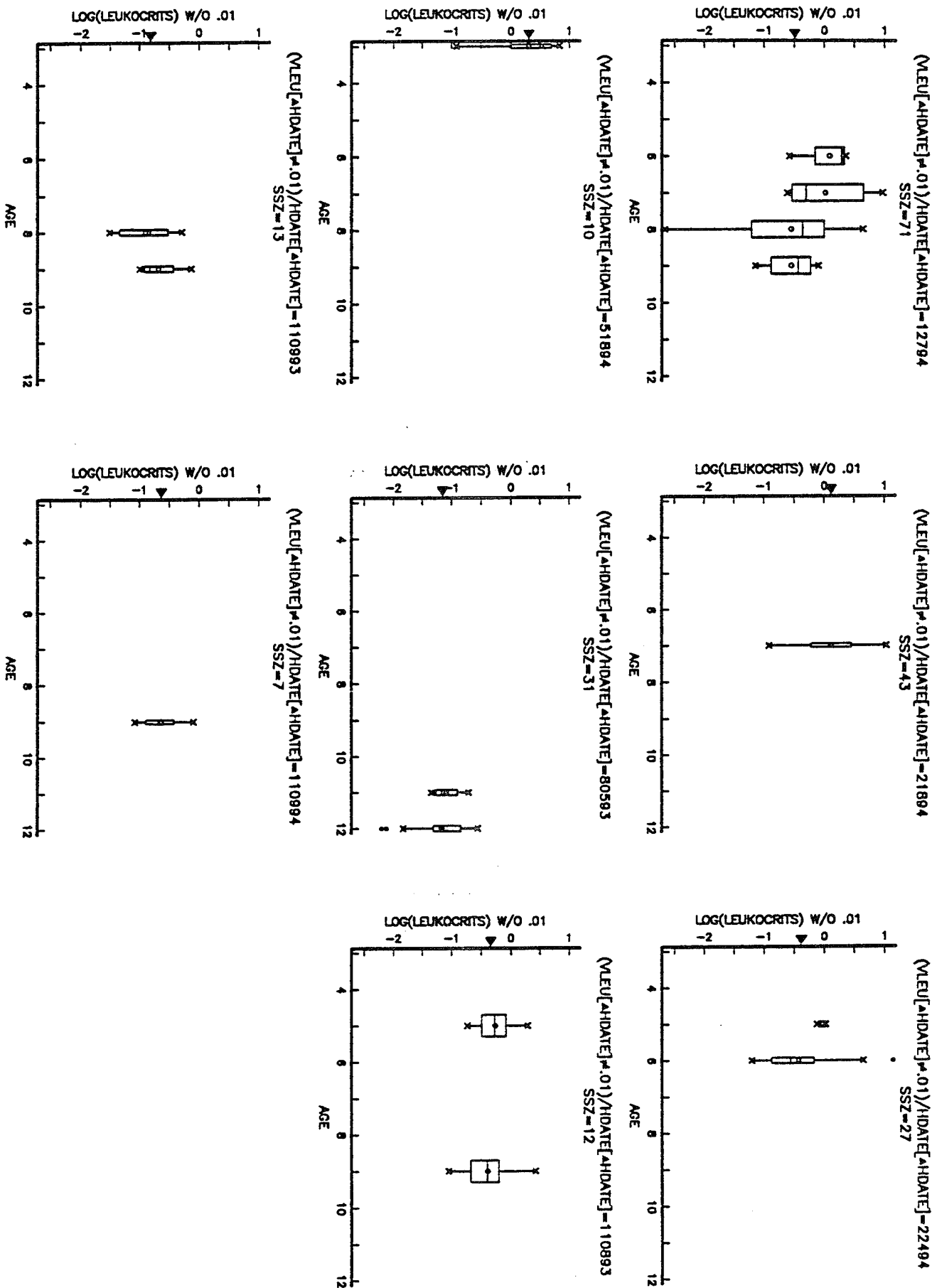


Figure 5

1 FIGURE FOR EACH HATCH DATE; 1 BOX PLOT FOR EACH AGE

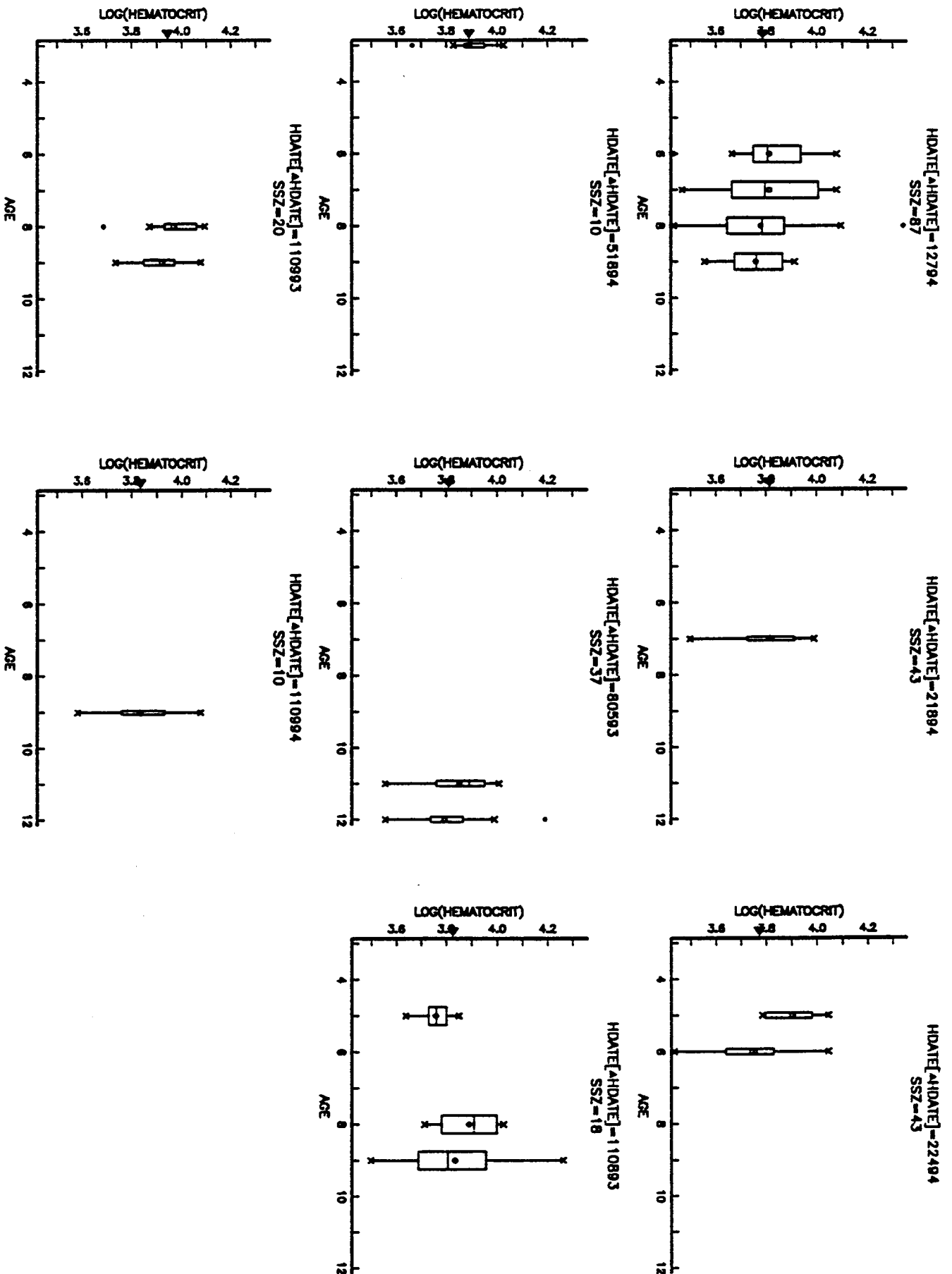


Figure 6

1 FIGURE BY HATCH DATE;1 BOX PLOT FOR EACH AGE

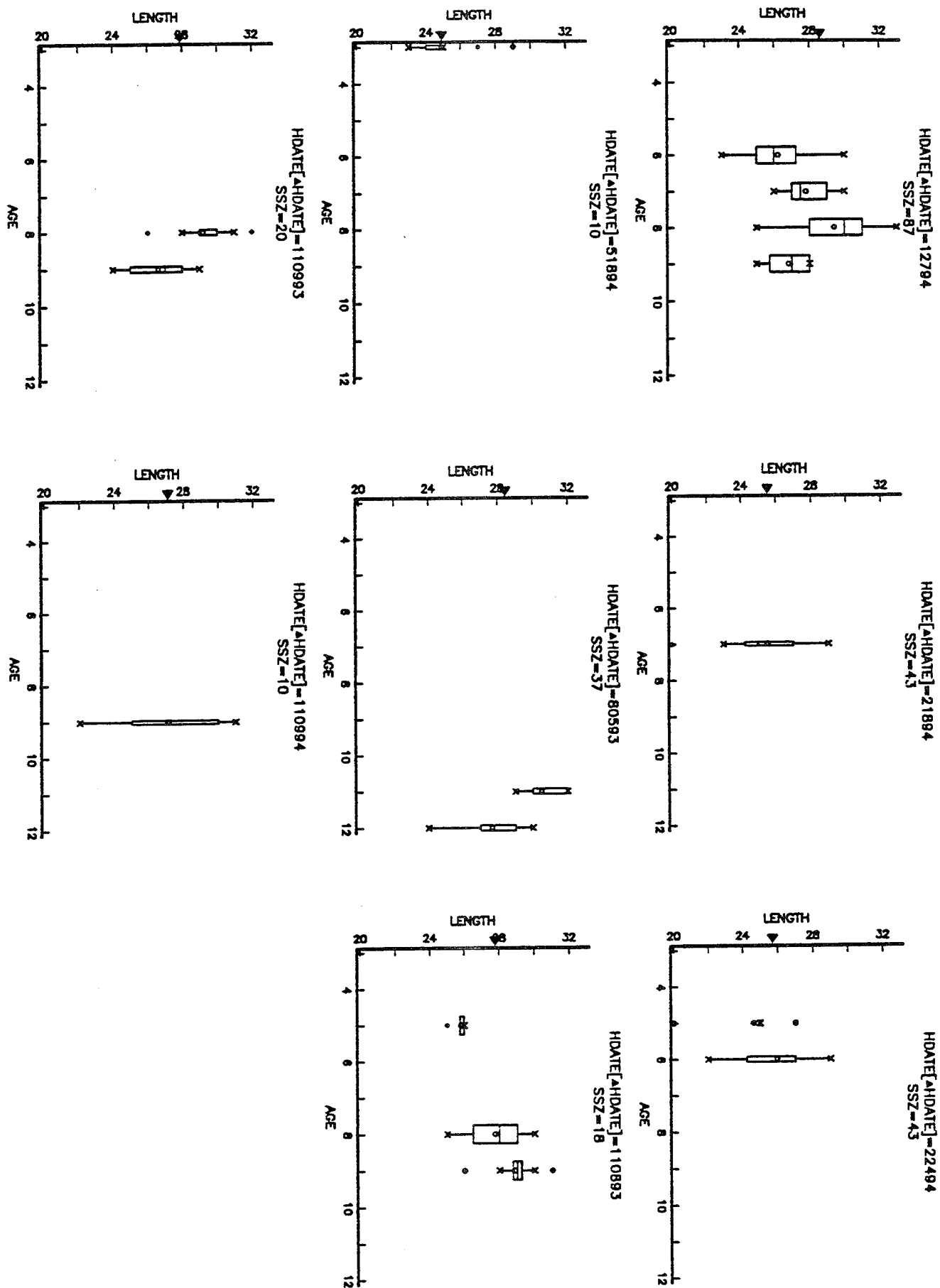


Figure 7

1 FIGURE BY HATCH DATE;1 BOX PLOT FOR EACH AGE

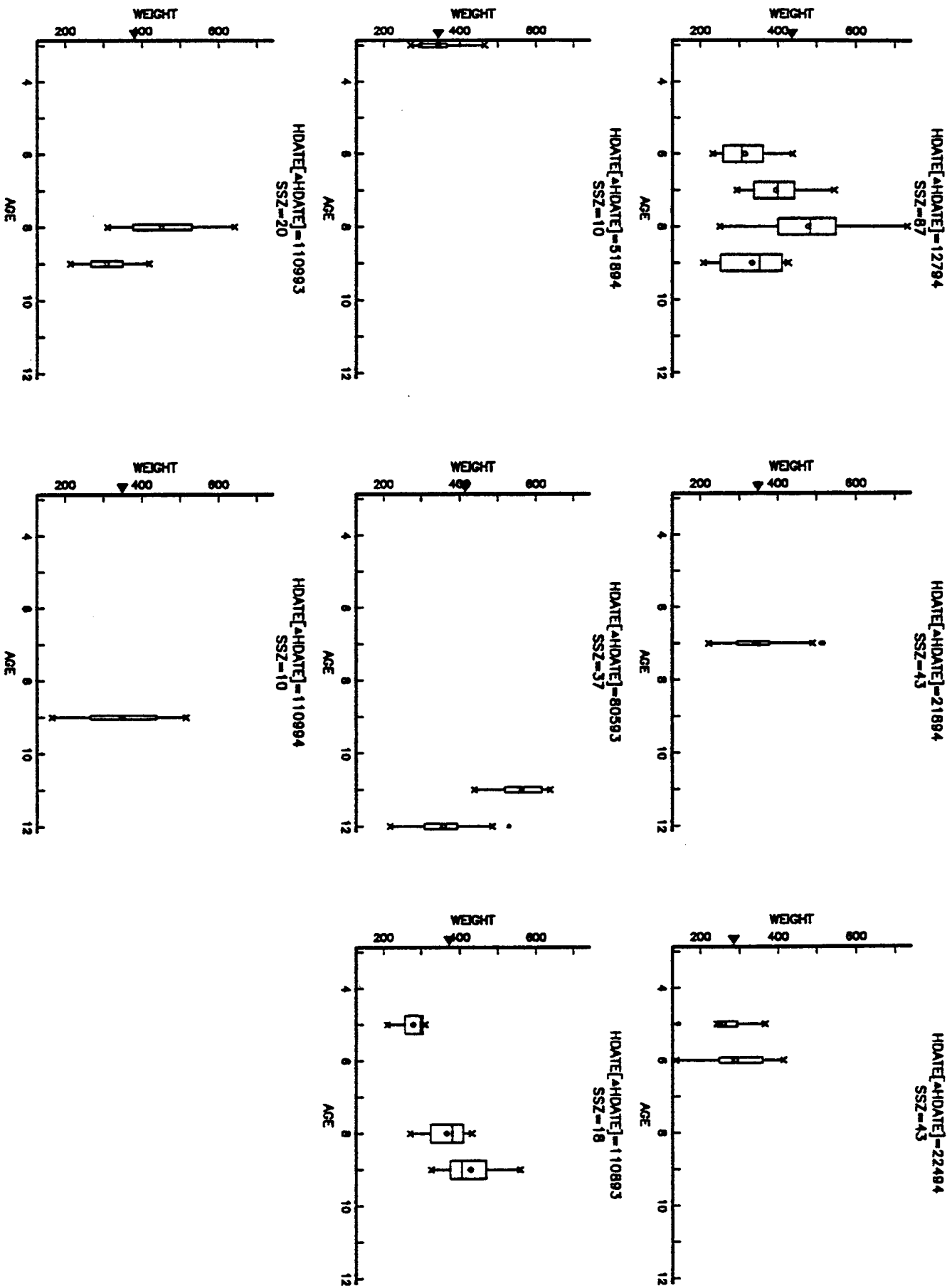


Figure 8

SCATTER PLOTS FOR SUBSAMPLES, SSZ=268

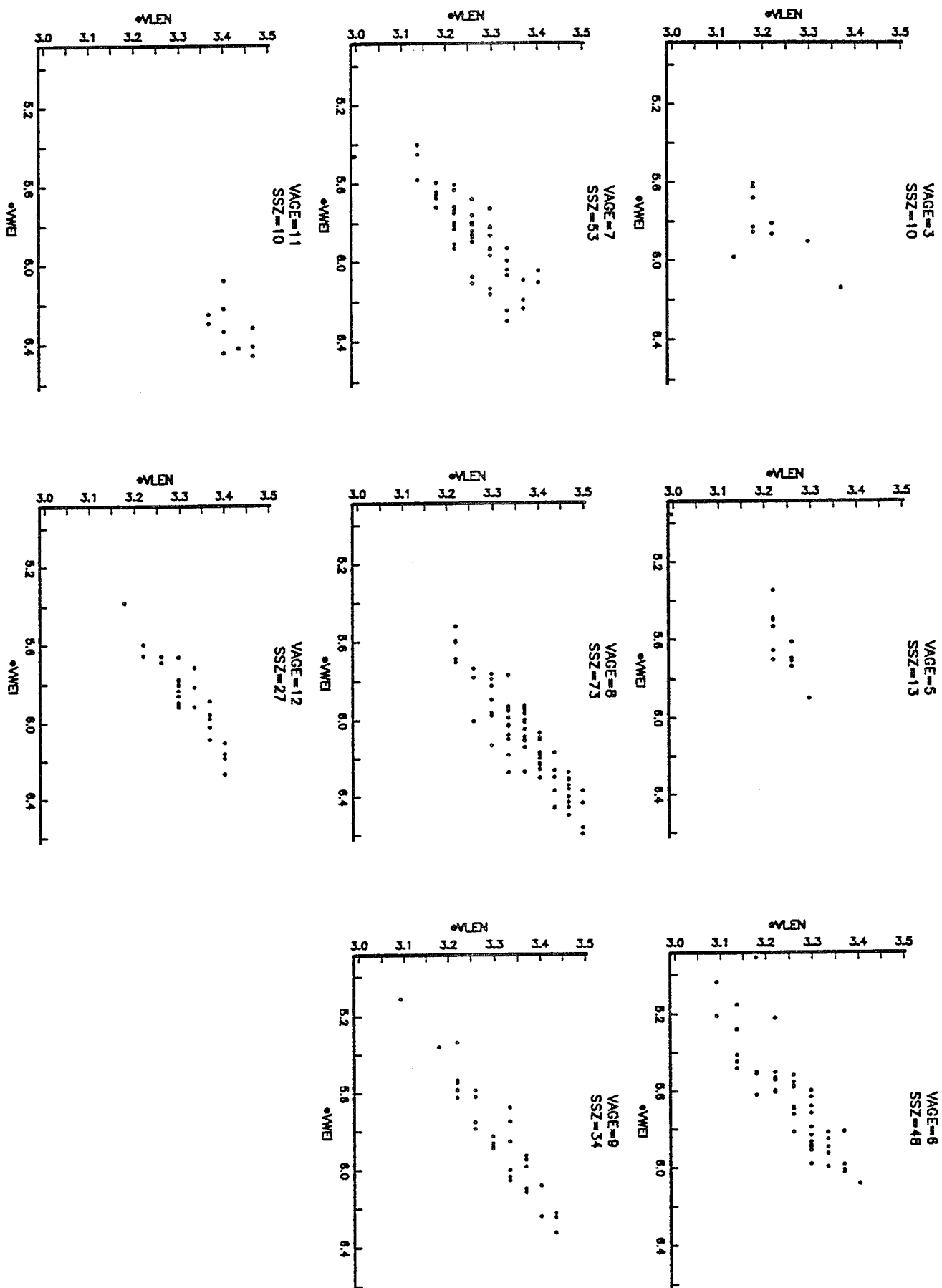


Figure 9

ONE SCATTER PLOT FOR EACH AGE;W/O LEU=.01

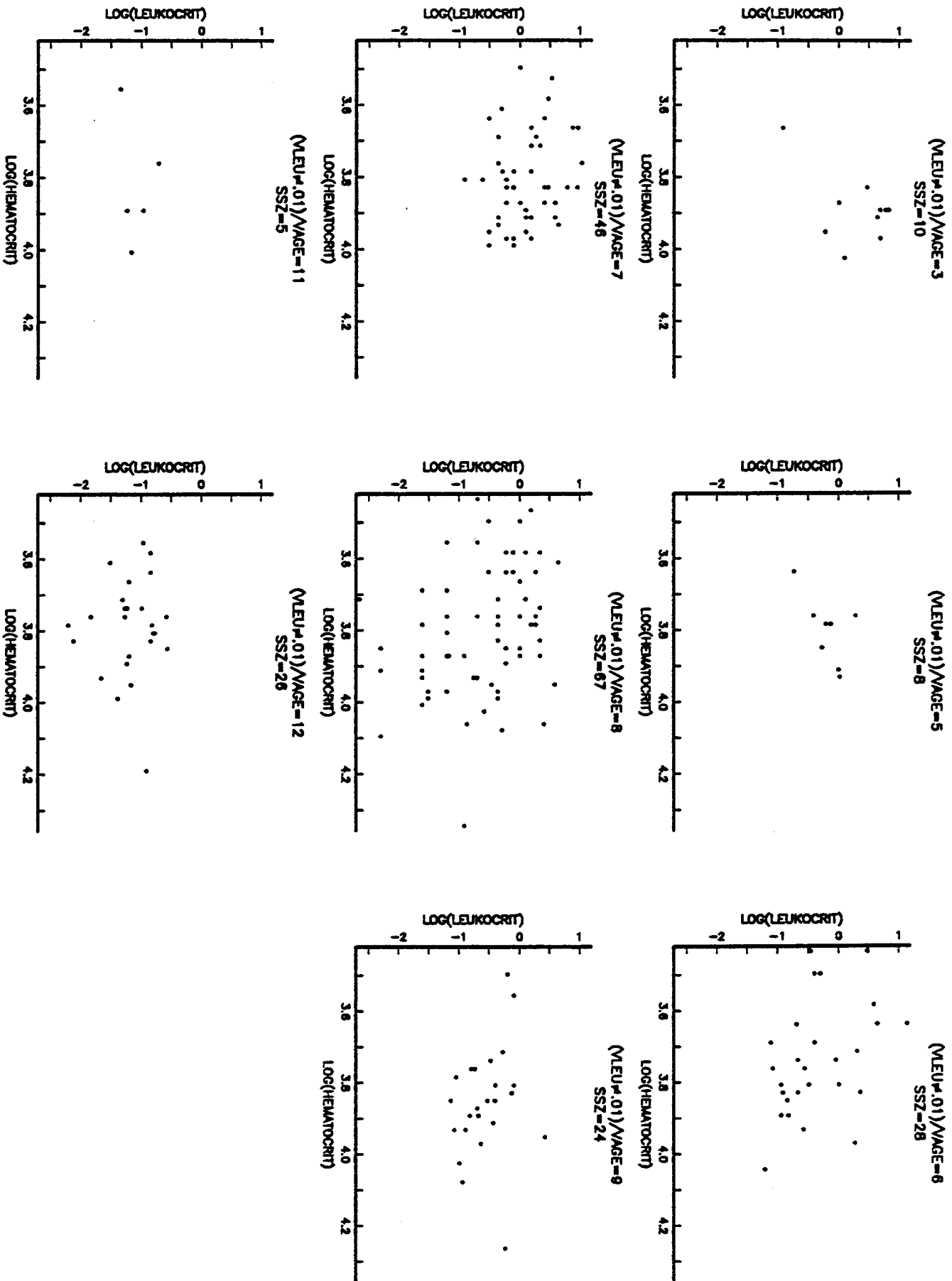


Figure 10

LOG(HEMATOCRIT) VS AGE

BOX/PERCENTILE PLOTS, SSZ=268

— QUARTILES
○ MEANS
× ADJACENT VALUES
○ OUTSIDE POINTS
● DETACHED POINTS

ANOVA DOES NOT REJECT NULL HYP OF EQUAL MEANS
 $F=1.7$ WITH DEGF BETW=7 AND DEGF WITHIN=260
 $p=0.89$

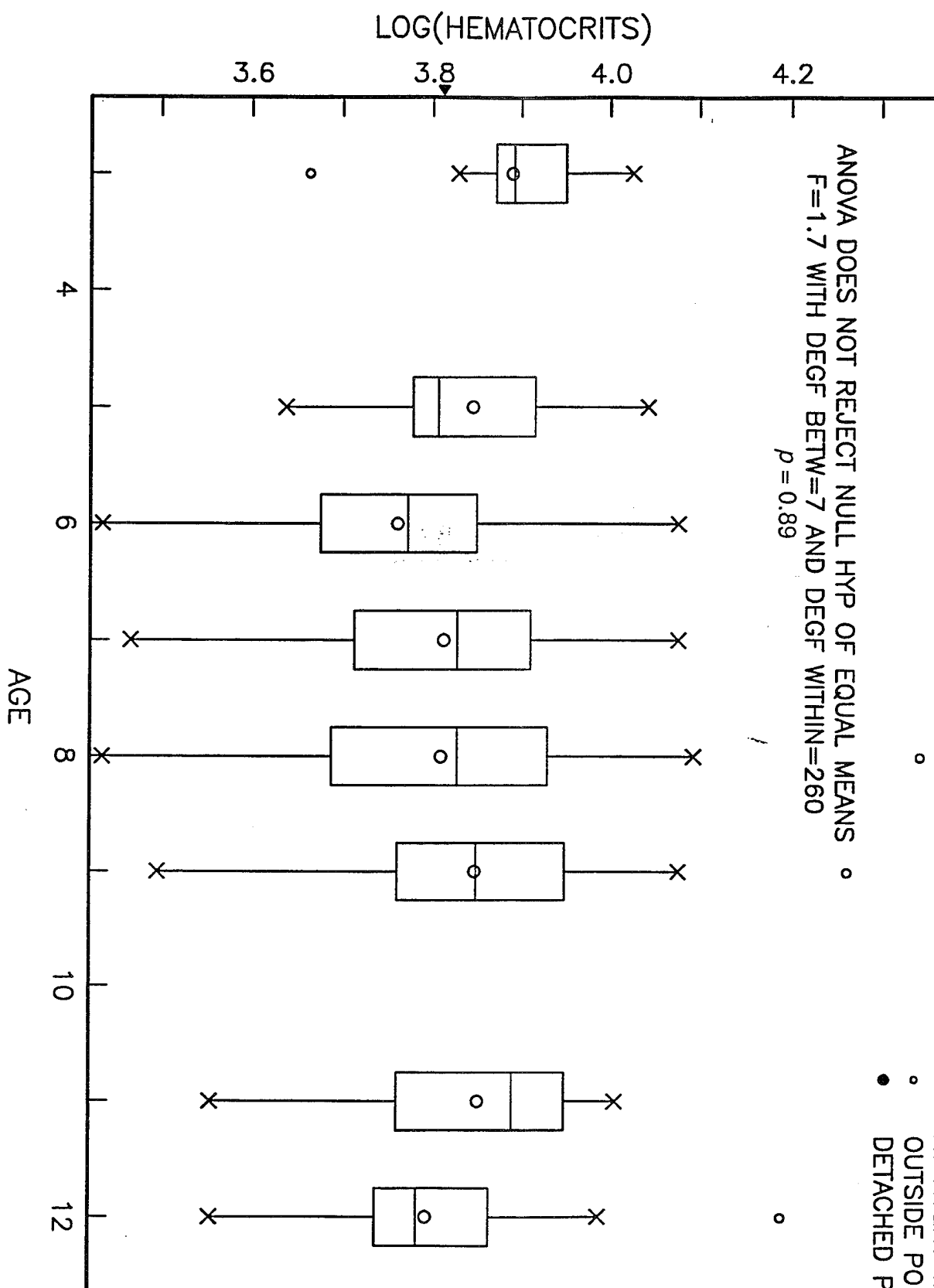
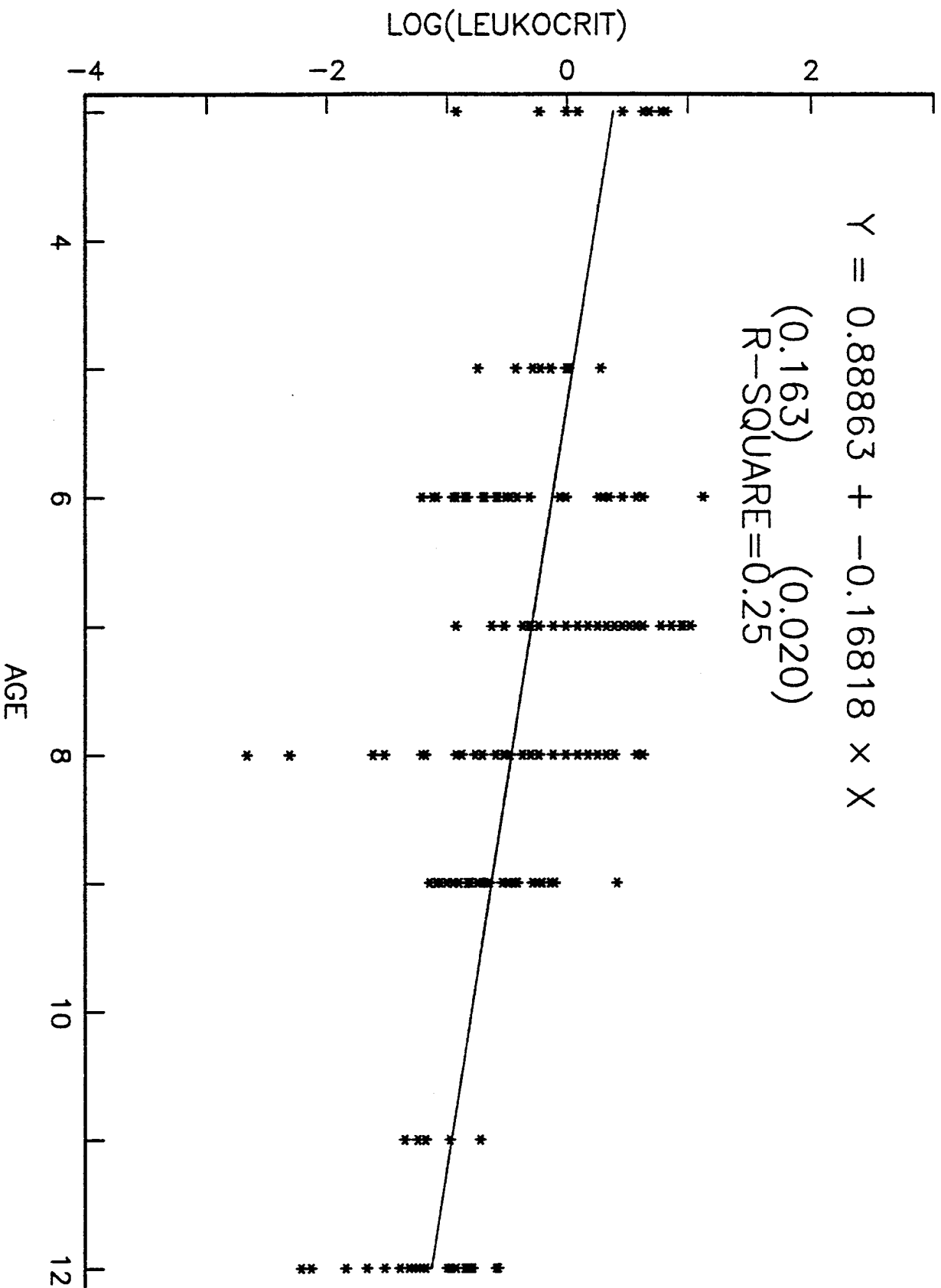


Figure 11

WITHOUT LEUKOCRIT=.01;DATA OF 10/94

LOG(LEUKOCRIT) VS AGE



LOG(LUEKOCRIT)-(.89-(.17×AGE));W/O .01 LEUK

BOX/PERCENTILE PLOTS, SSZ=214

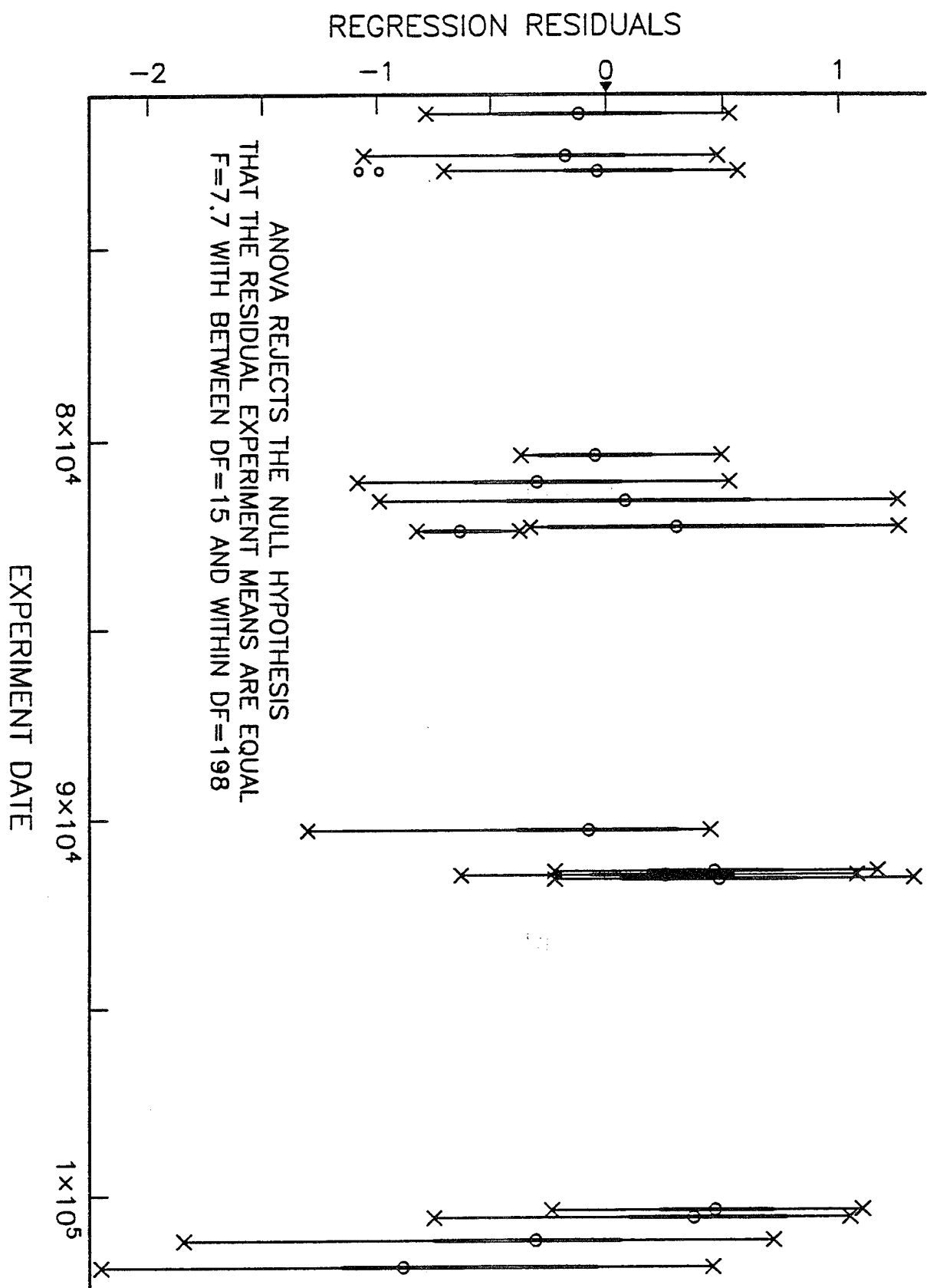


Figure 13

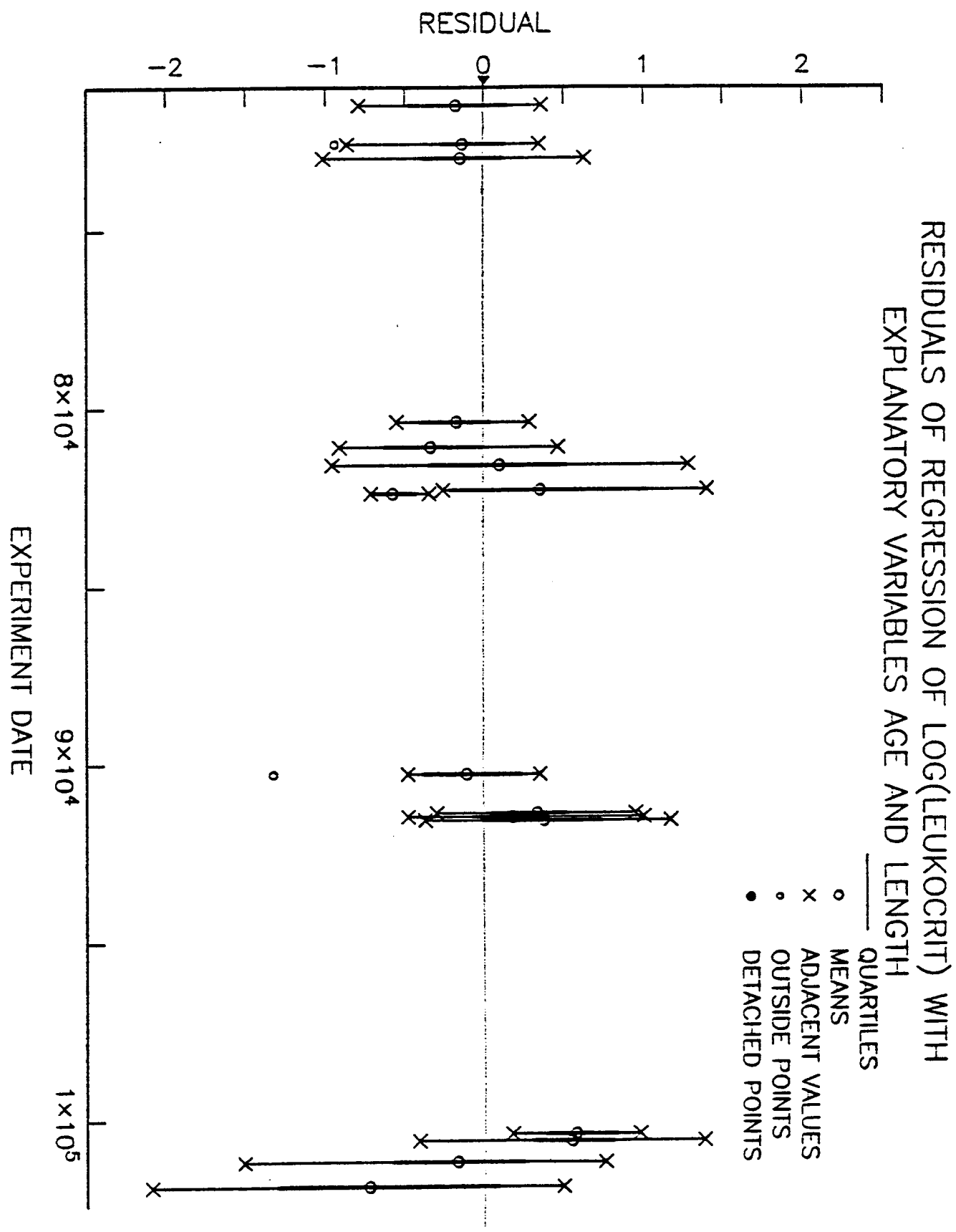


Figure 14

APPENDIX 7

MATHEMATICAL MODELING OF FREE RADICAL ($O_2^{\cdot-}$) PRODUCTION STIMULATION BY PMA

D. P. Gaver

P. A. Jacobs

1. Purpose.

The objective of this note is to provide a preliminary simple mathematical model to describe, in a quantitative way, the behavior of data exhibited by Dr. Judith Zelikoff at a recent USABRDL Research Project Review (Sept. 20-21, 1994). The data, shown in graphical form, appeared qualitatively as follows:

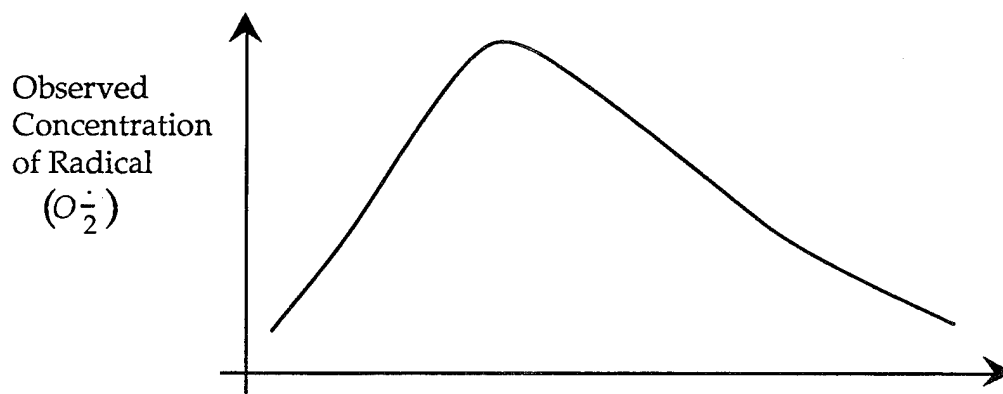


FIGURE 1

The radical production was stimulated by the introduction of an initial dose of PMA at time zero; the resulting production was made visible or observable by surrounding the cells so exposed with a solution of Luminal.

It was shown experimentally that quantitative features of the above dose response curve changed with experimental conditions such as the PMA dose

(presumably measured by its concentration and the amount of solution introduced, plus other experimental features and conditions).

2. Simplest Mathematical Model.

Denote the various quantities present as follows:

$P(t)$ = amount of PMA (or perhaps another substance, such as a toxicant or chemical) present at time t that has not yet interacted with a cell;

$R(t)$ = amount of radical, $O_2^{\cdot -}$, present, the appearance of which has been caused by PMA;

C = constant cell population size.

Here is a flow diagram for the interaction envisioned:

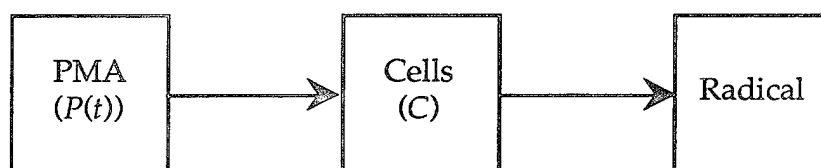


FIGURE 2

It is understood that when the radical is formed it becomes (bio)luminescent in the presence of luminal; this material is present at all times and does not change in concentration. The luminescence is assumed to be proportional to $R(t)$ (this may not be strictly correct, J.Z. to modify any of the above).

First Model.

If it is assumed that the cell population is large, and essentially unchanged by interaction with PMA, then the latter, which is introduced in amount $P(0)$ at $t = 0$, reduces as follows in time $(t, t + \Delta)$:

$$P(t + \Delta) = P(t) - \lambda P(t) C \Delta. \quad (1)$$

Passing to the limit as $\Delta \rightarrow 0$ this differential equation results

$$\frac{dP}{dt} = -\lambda CP(t) \quad (2)$$

which gives

$$P(t) = P(0)e^{-\lambda Ct}. \quad (3)$$

Next consider radical production and "death", i.e. the presumed cessation of radical production by a cell after initial stimulation.

$$R(t + \Delta) = R(t) + \underbrace{\lambda P(t)C \Delta}_{\text{Radical Production}} - \underbrace{\mu R(t)\Delta}_{\text{Radical Reduction in time; "death"}}. \quad (4)$$

This gives

$$\begin{aligned} \frac{dR}{dt} &= \lambda P(t)C - \mu R(t) \\ &= \lambda CP(0)e^{-\lambda Ct} - \mu R(t). \end{aligned} \quad (5)$$

This has the solution (by standard integrating factors)

$$R(t) = R(0)e^{-\mu t} + \frac{P(0)\lambda C}{\lambda C - \mu} \left(e^{-\mu t} - e^{-\lambda Ct} \right). \quad (6)$$

Very possibly, $R(0)$, the initial number of luminescent cells, is negligible, i.e. $R(0) = 0$. In this case

$$R(t) = \frac{P(0)\lambda C}{\lambda C - \mu} \left(e^{-\mu t} - e^{-\lambda Ct} \right). \quad (7)$$

The graph of this function appears much like Figure 1; the time at which a maximum is reached can be derived (differentiate and equate to zero and solve):

$$t_m = \frac{1}{\lambda C - \mu} \ln \left(\frac{\lambda C}{\mu} \right) \quad (8)$$

the height of the maximum, $R(t_m)$, is obtained by substitution:

$$R(t_m) = \frac{P(0)\lambda C}{\lambda C - \mu} \left[\left(\frac{\mu}{\lambda C} \right)^{\frac{\mu}{\lambda C - \mu}} - \left(\frac{\mu}{\lambda C} \right)^{\frac{\lambda C}{\lambda C - \mu}} \right]. \quad (9)$$

This formula must be studied numerically.

Second Model (Stochastic/Probabilistic Version of First). Towards Likelihood Estimation of Parameters.

Suppose we think of the setup as that of a number of PMA “particles” wandering in the cell vicinity at random. Assume that a particle avoids a cell for time t with probability $e^{-\lambda t}$. If it avoids all cells independently then the probability that it does not hit any of the C cells in time t is $(e^{-\lambda t})^C$. If $P(0)$ is the initial number of PMA particles introduced, then, assuming independence of particles, the *mean* number of still-wandering particles at time t — those that have not yet hit cells and initiated radical production — is $P(0)e^{-\lambda t}$, which is exactly $P(t)$ as calculated in (3).

The probability density of time for a PMA collision with a (some) cell is

$$f(t) = e^{-\lambda C t} \lambda C.$$

Suppose that a cell remains bioluminescent after collision for time y with probability $e^{-\mu y}$. Consequently the probability that a single cell is bioluminescent at any time t is the probability that (a) the cell is hit by a PMA particle at $x < t$, and (b) remains bioluminescent for time $t - x$; the latter must be “added up” or integrated over $0 \leq x \leq t$!

$$\begin{aligned} \text{Prob}(\text{Cell is biolumin. at time } t) &= \int_0^t f(x) e^{-\mu(t-x)} dx = \int_0^t e^{-\lambda C x} \lambda C e^{-\mu(t-x)} dx \\ &= \frac{\lambda C}{\lambda C - \mu} (e^{-\mu t} - e^{-\lambda C t}). \end{aligned}$$

The *mean* number of bioluminescent cells is thus

$$\text{Mean Number Bioluminescent Cells at } t = \frac{P(0)\lambda C}{\lambda C - \mu} (e^{-\mu t} - e^{-\lambda C t})$$

which is precisely what was obtained earlier in (7). A bonus is that according to this model (which may correspond only approximately to reality) the actual random number of bioluminescent cells at t , $R(t)$, is binomially distributed with trial number $P(0)$ and probability of bioluminescence

$$b(t) = \frac{\lambda C}{\lambda C - \mu} (e^{-\mu t} - e^{-\lambda C t}).$$

This allows one to write down formal statistical estimation equations (maximum likelihood method) for parameters such as λ , μ and perhaps an "effective $P(0)$ ". Having these would provide a quantitative dose-response relationship.

Design of Observations.

It appears likely that observations or measurements of bioluminescence will be made at discrete intervals of time, possibly at

$$t_1 < t_2 < t_3 < \dots < t_k$$

at which times we would get corresponding measured values $r(t_1) = r_1$, $r(t_2) = r_2$, ...etc. Question: if the times, t_i , can be chosen deliberately, what should their values be? Choice of certain values will give better parameter estimates than others; in other words we seek to optimize (minimize) estimation error within the constraints of time and experimental cost. This can be studied quantitatively, perhaps by simulation, in advance of actual simulation, provided rough values for the parameters are available. This problem and opportunity will be discussed in more detail later.

APPENDIX 8

MODELS FOR ADDUCT DAMAGE FIXATION IN THE PRESENCE OF REPAIR

Donald P. Gaver
Patricia A. Jacobs

1. Problem Formulation

Suppose an idealized organ, e.g. liver, is made up of originally homogeneous elements, i.e. cells, that cycle (die and replicate) under the (temporary) constraint that their number is fixed. Let them initially and thereafter be subject to a dose stimulus that tends to change normal cells to adduct-ridden cells; thus at time t there are $D_o(t)$ normal cells and $D_a(t)$ adducted cells, and $D_o(t) + D_a(t) = C$, C being (temporarily) a constant, e.g. $\sim 10^{10}$ as in human liver.

Assume that the presence of the adducted cells induces the normal cells to supply a repair agent that effectively produces an enhanced adducted cell death rate, i.e. stimulates apoptosis. This is the first step in population repair. What is the behavior of the resulting population of adducted — and hence also normal — cells?

An extremely simple — probably vastly over-simplified — mathematical model is suggested, followed by some comments and alternatives. There are many! Suggestions are solicited.

2. Specific Mathematical Details: Model I

Let $R(t)$ denote the concentration of the specific adducted cell enemy. Perhaps it is alkyl-DNA transferase “capable of removing ethyl groups from the O⁶-position of guanine ...”, see Burkhart and Malling (1993).

Model I

Now assume that the net rate of change of $R(t)$ is

$$\boxed{\frac{dR(t)}{dt} = \rho D_a(t) - \delta_r R(t)} \quad (2.1)$$

We call the model incorporating (2.1) Model I. The first, leftmost, term on the right-hand side (tries to) represents the rate of production of repair-inducing agent; the second represents the rate at which that material leaves the organ either by metabolism or binding or transfer out by blood circulation or ...?

- **Comments on (2.1): There Are Many Alternatives**

Mathematical modeling forces specificity, which stimulates experimentation to determine more likely alternative mechanisms. The above equation is therefore tentative, being precisely wrong but perhaps usefully adequate.

(a) The repair material creation term, $\rho D_a(t)$, may be plausible if it is viewed as a pure source term from adducted cells. But what if adducted cells are signalling non-adducted = normal cells to produce the material? This may well be more biologically plausible and interesting, and its consequences will be investigated subsequently. Then a more appropriate term might be $\rho D_a(t) D_o(t) = \rho D_a(t)[C - D_a(t)]$; the second term of the new expression (qualitatively) represents the possibility that if $D_a(t)$ gets large, there are no recipients for a signal, and the adduct repair or healing agent cannot be produced. Presumably the organ is now in a more endangered state. We call the model that replaces $\rho D_a(t)$ by $\rho D_a(t)[C - D_a(t)]$ Model II, and take it up later.

(b) If adducts occur in a clonal, packed, condition then there is the possibility that *linear* dependence of $D_a(t)$ in repair creation or signaling is

inappropriate: a better model might be $D_a^p(t)$, where $p < 1$, e.g. 1/2 or 1/3 to represent something like surface-exposed adducted cells.

The second equation given is for the net rate of adducted-cell increase:

$$\frac{dD_a(t)}{dt} = \underbrace{\lambda_{oa}[C - D_a(t)]}_{\text{Recruitment of Normal Cells to Adducted}} + \underbrace{\lambda_{aa}D_a(t)}_{\text{Adducted Cell Birth}} - \underbrace{\delta_a D_a(t)}_{\text{Adducted Cell Death}} - \underbrace{\delta_{ar}R(t)D_a(t)}_{\text{Adducted Cell Death, Hence "Repair" of Tissue}} \quad (2.2)$$

The notations below the terms represent the — hypothetical — effects modeled by each term. Again there are more alternatives.

• **Comments on (2.2)**

(a) One can allow all parameters of the adduct-cell changes to be *time*, in particular organ *age*, dependent. If the organ grows it is presumably mostly in the context of a young host animal becoming older. This is inconsistent with a constant C-value. Growing organs will be modeled later.

(b) Dimensional or morphological considerations may well force changes. For example, the repair term, $\delta_{ar}R(t)D_a(t)$, might be better replaced by $\delta_{ar}R(t)D_a^p(t)$, and $\lambda_a D_a(t)$ by $\lambda_a D_a^p(t)$ in Model I if clonal expansion is a surface effect.

(c) In neither (2.1) nor (2.2) has any attention been given to physical dimensions, except implicitly. $R(t)$ and $D_a(t)$ are, of course, not of the same dimension.

3. Implications

Equations (2.1) and (2.2) are first-order non-linear differential equations that can be solved numerically. Such solutions are the only recourse if parameters are

time dependent. However, some information can be obtained in parametric form under certain conditions.

- **Chronic Exposure and Steady State**

Suppose a toxic agent that affects λ_{oa} , the exogenously generated toxic exposure, is constant over time. This should mean that λ_{oa} is also constant. Assume also that all other rates are constant. Then when time grows long ($t \rightarrow \infty$) a steady-state fraction of adducted cells will result, as will a steady-state concentration of adduct repair agent; let these be the constants D_a and R respectively. They satisfy the equations (2.1)' and (2.2)' respectively, which are (2.1) and (2.2) with left-hand side derivatives equated to zero. Immediately $R = (\rho/\delta_r)D_a$ from (2.1)' and D_a now satisfies the quadratic equation

$$0 = \lambda_{oa}[C - D_a] + \lambda_{aa}D_a - \delta_a D_a - \delta_{ar}(\rho/\delta_r)D_a^2. \quad (3.1)$$

This can be explicitly solved to give a formula for the solution, D_a , involving all parameters: solve (3.1) to obtain the formula

$$d_a = \frac{2\lambda_{oa}C}{(\delta_a - \lambda_{aa} + \lambda_{oa}) + \sqrt{(\delta_a - \lambda_{aa} + \lambda_{oa})^2 + 4\delta_{ar}(\rho/\delta_r)\lambda_{oa}C}};$$

then

$$D_a^* = \begin{cases} d_a & \text{if } 0 \leq d_a \leq C \\ 0 & \text{if } d_a < 0 \\ C & \text{if } d_a > C. \end{cases}$$

Insight is furnished by a graphical display. Write (3.1) as "birth-vs-death":

$$[\lambda_{oa}(C - D_a)] + \lambda_{aa}D_a = \delta_a D_a + \delta_{ar}(\rho/\delta_r)D_a^2 \quad (3.2a)$$

or, for convenience,

$$L(D_a; \lambda_{oa}, \lambda_{aa}) = R(D_a, \delta_a, \delta_{ar}, \rho, \delta_r) \quad (3.2b)$$

and plot the left-hand side and the right-hand side, vs. D_a , mindful of the constraint $0 \leq D_a \leq C$. It is helpful to plot the bracketed term, $[\cdot]$, of (3.2a) and the term $+\lambda_{aa}D_a$ separately to see that the net effect of increasing either/both λ_{oa} , adducted cell recruitment rate, and λ_{aa} , adducted cell reproduction or growth rate increases the $L(D_a; \cdot)$ at every D_a -value. The solution is at $L(D_a^*) = R(D_a^*)$, where the curves cross; otherwise the solution is at C .

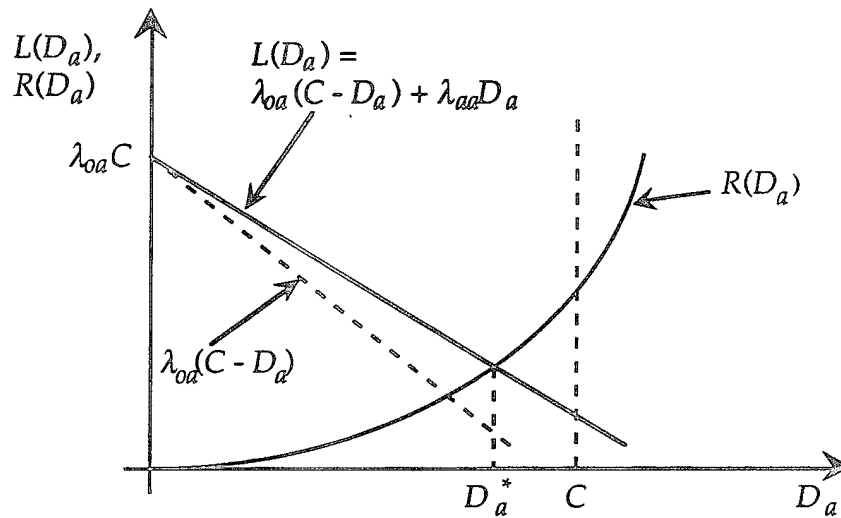


Figure 1

Since $R_a(D_a)$ is an upward rising parabola there will always be a solution value, D_a^* inside $[0, C]$ unless $R(C) < L(C)$, in which case the organ is eventually taken over by adducts, or $D_a^* = C$. (Before this occurs other, more serious, events presumably befall the animal host, but these are not modeled.) A brief check easily shows that D_a^* is, *ceteris paribus*, monotonically increasing in λ_{oa} and λ_{aa} separately. On the other hand, the parabolic $R(D_a)$ shifts higher if δ_a , the background adducted cell death rate, and δ_{ar} , the induced extra death rate, both increase; either change monotonically decreases D_a^* . The above effect also results if ρ , the rate of production of repair agent, increases; D_a^* also increases with

reduction of δ_r , the rate at which the effect of $R(t)$ diminishes. All effects are intuitive, taken individually.

Note, however, that the effect of a chemical toxin in the vicinity of the organ's cells, not explicitly modeled, may *simultaneously* affect the various parameters above in quite different ways, possibly increasing λ_{aa} and λ_{oa} as might be expected, but also possibly increasing the creation of ρ , say, more than enough to compensate so that the adducted-cell response to toxin, T , $D_a^*(T)$, could actually *reduce* for some range of T ; possibly ultimately the cell repair capacity would fail and the toxin would win out. The above mechanism could easily create a *non-monotonic dose-response* function, or give rise to the elusive "hormesis" phenomenon. The same effect might be achieved by reducing δ_r , the rate of repair agent removal. This suggests the importance of understanding the effect of a chemical toxin on all components of the system governing mechanism.

4. Approximate Time-Dependent Behavior of Model I

It is of interest to trace the development of the adducted cell population as it evolves in time. This can be done explicitly if the *quasi-stationary assumption* for $R(t)$ is satisfied, i.e. if owing to relatively fast interaction of R with D_a we can assume in (2.1) that $\frac{dR(t)}{dt} \equiv 0$. Such an approach has been studied carefully by Segel and Slemrod (1989); it is used to justify the familiar Michaelis-Menton formula automatically invoked in much pharmacokinetic work. If this assumption is assumed valid we obtain the *single* non-linear differential equation for $D_a(t)$:

$$\frac{dD_a}{dt} = \lambda_{oa}[C - D_a(t)] + \lambda_{aa}D_a(t) - \delta_a D_a(t) - \delta_{ar}(\rho/\delta_r)D_a^2(t). \quad (4.1)$$

The above is recognized as a non-homogeneous Riccati equation that admits an explicit solution; the latter will approach the previous long-run solution,

provided one is mindful of the constraint $0 \leq D_a(t) \leq C$. Note that this constraint must be imposed because of the possible influence of the positive term $\lambda_{aa}D(t)$ representing an adducted cell proliferation process that does not recognize the inhibiting presence of the organ boundary at C . This can be made gentle or soft, or *natural*, but in fact the model is not expected to apply when $D_a(t)$ is anywhere near organ size: long before that, the host would be dead.

• **A Toxic Bolus Dosage: An Initial Number of Adducted Cells Given**

It is common to ask for the effect of an initial chemical bolus dosage, or single acute exposure. This outcome can be modeled by removing the first right-hand side source term, $\lambda_{aa}[C - D_a(t)]$ of (4.1) and solving the resulting homogeneous equation, where the initial condition $D_a(0)$ is the adduct load delivered by the initial dose. An explicit if complicated-appearing solution appears:

$$D_a(t) = \frac{K_1 e^{(\lambda_{aa} - \delta_a)t}}{1 + K_2 e^{(\lambda_{aa} - \delta_a)t}}. \quad (4.2)$$

See Appendix 1 for the explicit forms of K_1 and K_2 . Note that in the present situation the behavior of $D_a(t)$ can *either* increase initially and later drop to zero, with a single maximum at t_m ($t_m > 0$), where

$$D_a(t_m) = (\lambda_{aa} - \delta_a) / \delta_{ar} (\rho / \delta_r)$$

if $\lambda_{aa} > \delta_a$, or otherwise drop monotonically to zero if $\lambda_{aa} - \delta_a - \delta_{ar}(\rho / \delta_r)D_a(0) < 0$.

If desired a single overall summary measure of adduct cell presence, a *response* to the chemical dosage giving $D_a(0)$, can be obtained by integrating (4.1), which can be carried out explicitly. This step is omitted for the moment.

5. Model II; An Alternative That Emphasizes Signaling

It was noted earlier that a quite plausible alternative model for induction of repair agent R appears as attempts to model the effect of cell signalling

$$\frac{dD_a(t)}{dt} = \lambda_{oa}(C - D_a(t)) + \lambda_{aa}D_a(t) - \delta_a D_a(t) - \delta_{ar}(\rho/\delta_r)D_a^2(t)(C - D_a(t)). \quad (5.1)$$

See Section _____, paragraph _____ for discussion.

• Steady-State: Possible Bistability

Here let D_a be the long-run mean number of adducted cells as before and

$$\begin{aligned} L(D_a; \lambda_{oa}, \lambda_{aa}) &\equiv \lambda_{oa}(C - D_a) + \lambda_{aa}D_a = \delta_a D_a + \delta_{ar}(\rho/\delta_r)D_a^2(C - D_a) \\ &\equiv R(D_a; \delta_a, \delta_{ar}, \rho, \delta_r, C) \end{aligned} \quad (5.2)$$

while the graph of $L(D_a)$ remains the same that for $R(D_a)$ changes:

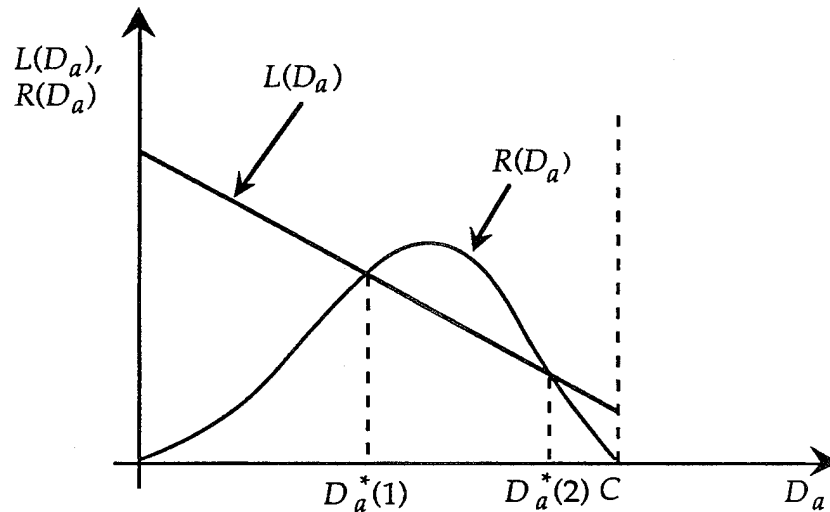


Figure 2

Now if $D_a(t) < D_a^*(1)$ then $L(D_a(t)) > R(D_a(t))$ and the derivative $\frac{dD_a(t)}{dt} > 0$, moving $D_a(t)$ towards $D_a^*(1)$. If $D_a^*(1) < D_a(t) < D_a^*(2)$, $\frac{dD_a(t)}{dt} < 0$, reducing the value of $D_a(t)$ towards $D_a^*(1)$. Hence $D_a^*(1)$ is a stable equilibrium point, reached eventually by any value of $\{D_a(t), t > 0\}$ that starts with $D_a(t) < D_a^*(2)$. On the

other hand, if $D_a^*(2) < D_a(t) \leq C$ the derivative $\frac{D_a(t)}{dt}$ is always positive and the process gravitates towards $D_a(\infty) = C$ in the long run. There are thus *two* stable points or long-run (mean) numbers of adducted cells: a (comparatively) low value, here $D_a^*(1)$, and a high value, $D_a^* = C$. In the present simple theory either one can be reached, depending upon the initial conditions (and the parameter values in effect): $0 \leq D_a(0) \leq D_a^*(2)$ guarantees that $D_a(t) \rightarrow D_a^*(1)$, wandering throughout $[0, D_a^*(2)]$ while doing so; $D_a^*(2) < D_a(0) \leq C$ assumes that the number of adducted cell eventually takes over the organ. This may perhaps imply the existence of a threshold with respect to response to $D_a(0)$, itself the effect of dosage: *ceteris paribus* "small" toxin doses, i.e. giving $D_a(0) < D_a^*(2)$, will induce "small" numbers of adducted cells that remain low in number and of little threat, whereas "large" doses, with $D_a(0) > D_a^*(2)$ will tend to foster an ever increasing adducted cell population in the organ.

6. A Stochastic Version of the Process

There are many chance-affected elements of the above process capable of making it stochastic. For instance, differences between replicate animals might be represented by random choice of all governing parameters from suitable joint distributions, or dose or exposure itself may vary randomly around a desired level. But a natural first step is to simply suppose that the elementary processes such as adducted cell import, birth, and death are "random" in the sense of birth-and-death processes; cf. Feller (1968) or Karlin and Taylor (1975).

• Naive Conversion to Birth-Death

Let now $D_a(t)$ be the random state variable of a one-dimensional Markov process, and *define* the generator as follows, using the terms of (5.1) for guidance. Given $D_a(t)$, write

$$P\{D_a(t+dt) = d+1 | D_a(t) = d\} = [\lambda_{0a}(C-d) + \lambda_{aa}d]dt \equiv \lambda_d dt \quad (6.1)$$

and

$$P\{D_a(t+dt) = d-1 | D_a(t) = d\} = [\delta_a d + \delta_{ar}(\rho/\delta_r)d^2(C-d)]dt \equiv \mu_d dt. \quad (6.2)$$

The fact that the transition functions are non-linear means that the expected value of the random quantity $D_a(t)$ will not satisfy the deterministic equation (5.1) is caused by the inevitable higher moments. One trick useful for "closing off" this annoying "moment-creep" is to replace the higher moments by their equivalent for a Gaussian process; see Whittle (1957), and Isham (1991). This method often works well.

Neglect the above problem for simplicity and write down the stationary distribution of the Markov chain in continuous time that portrays $D_a(t)$ as specified. There clearly is such a stationary distribution (finite state space, all states communicate)

$$\pi_d = \pi_0 \frac{\lambda_0 \cdot \lambda_1 \cdot \dots \cdot \lambda_{d-1}}{\mu_1 \cdot \mu_2 \cdot \dots \cdot \mu_d} = \pi_{d-1} \cdot \frac{\lambda_{d-1}}{\mu_d}$$

References

- Burkhart and Mallin ... 1993.
- Feller, W. *An Introduction to Probability theory and its Applications*, Vol. I, Third Edition, John Wiley & Sons, Inc. 1968.
- Isham, V. "Assessing the variability of stochastic epidemics," *Mathematical Biosciences*, 1991, **107**, pp. 209-224.
- Karlin, S. and Taylor, H. M. *A First Course in Stochastic Processes*, Second Edition, Academic Press, 1975.
- Segal, L. A. and Slemrod, M. "The quasi-steady state assumption: a case study in perturbation," *SIAM Review*, 1989, **31**, pp. 446-477.
- Whittle, P. "On the use of the normal approximation in the treatment of stochastic processes," *J. Royal Statist. Soc. (B)*, 1957, **19**, pp. 268-281.

Appendix 1

Explicit (Logistic) Form of Adducted Cell Population at Any Time t ; Chronic Exposure (Constant λ_{aa})

Separate variables in (4.1) without the term $\lambda_{aa}[C - D_a(t)]$

$$\frac{dD_a(t)}{\lambda_{aa}D_a(t) - \delta_a D_a(t) - \delta_{ar}(\rho/\delta_r)D_a^2(t)} = dt. \quad (A1.1)$$

Rewriting (A1.1)

$$dD_a(t) \left[\frac{A}{D_a(t)} + \frac{B}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)} \right] = dt \quad (A1.2)$$

or

$$dD_a(t) \left[\frac{A[\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)] + BD_a(t)}{D_a(t)[\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)]} \right] = dt. \quad (A1.3)$$

Equating coefficients results in the equations

$$A[\lambda_{aa} - \delta_a] = 1$$

$$A[-\delta_{ar}(\rho/\delta_r)] + B = 0.$$

Thus

$$A = \frac{1}{\lambda_{aa} - \delta_a} \quad (A1.4)$$

and

$$B = \frac{\delta_{ar}(\rho/\delta_r)}{\lambda_{aa} - \delta_a}. \quad (A1.5)$$

Therefore from (A1.2)

$$\frac{dD_a(t)}{D_a(t)} - \frac{dD_a(t)}{D_a(t) - \frac{(\lambda_{aa} - \delta_a)}{\delta_{ar}(\rho/\delta_r)}} = (\lambda_{aa} - \delta_a)dt \quad (A1.6)$$

and integration yields

$$\ln \left[\frac{D_a(t)}{D_a(0)} \right] - \ln \left[\frac{D_a(t) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho}{D_a(0) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho} \right] = (\lambda_{aa} - \delta_a) t \quad (A1.7)$$

or

$$\ln \left[\frac{D_a(t)}{D_a(t) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho} \right] - \ln \left[\frac{D_a(0)}{D_a(0) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho} \right] = (\lambda_{aa} - \delta_a) t$$

or

$$\frac{D_a(t)}{[D_a(t) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho]} = K_1 e^{\kappa t} \quad (A1.8)$$

where

$$K_1 = \frac{D_a(0)}{[D_a(0) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho]} \quad (A1.9)$$

and

$$\kappa = \lambda_{aa} - \delta_a. \quad (A1.10)$$

Consequently,

$$D_a(t) = [D_a(t) - (\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho] K_1 e^{\kappa t}$$

so

$$D_a(t) = \frac{[-(\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho] K_1 e^{\kappa t}}{1 - K_1 e^{\kappa t}} \quad (A1.11)$$

$$= \frac{[(\lambda_{aa} - \delta_a) \delta_r / \delta_{ar} \rho] K_1 e^{\kappa t}}{K_1 e^{\kappa t} - 1} \quad (A1.12)$$

if $C \geq D_a(t) \geq 0$, which is of the anticipated logistic form. We do not exhaustively examine all possible cases.

Appendix 2

Solution of the Homogeneous Riccati Equation

Let

$$\frac{dD_a}{dt} = \lambda_{aa}D_a(t) - \delta_a D_a(t) - \delta_{ar}(\rho/\delta_r)D_a^2(t) \quad (A2.1)$$

given $D_a(0) > 0$. Separate:

$$\frac{dD_a(t)}{\lambda_{aa}D_a(t) - \delta_a D_a(t) - \delta_{ar}(\rho/\delta_r)D_a^2(t)} = dt. \quad (A2.2)$$

Employ partial fractions:

$$\left[\frac{1}{D_a(t)} + \frac{\delta_{ar}(\rho/\delta_r)}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)} \right] dD_a(t) = (\lambda_{aa} - \delta_a)dt. \quad (A2.3)$$

Integrate both sides:

$$\ln \left[\frac{D_a(t)}{D_a(0)} \right] - \ln \left[\frac{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(0)} \right] = (\lambda_{aa} + \delta_a)t. \quad (A2.4)$$

Rearrange:

$$\ln \left[\frac{D_a(t)}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)} \right] = \ln \left[\frac{D_a(0)}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(0)} \right] + (\lambda_{aa} + \delta_a)t \quad (A2.5)$$

so

$$\frac{D_a(t)}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(t)} = \frac{D_a(0)}{\lambda_{aa} - \delta_a - \delta_{ar}(\rho/\delta_r)D_a(0)} e^{(\lambda_{aa} + \delta_a)t}. \quad (A2.6)$$